STUDIES ON HEAT SHOCK PROTEIN 70, ANTIOXIDANT STATUS, BIOCHEMICAL AND HORMONAL PROFILES DURING SUMMER STRESS IN HALLIKAR CATTLE

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CERTIFICATE

This is to certify that the thesis entitled ***"STUDIES ON HEAT SHOCK PROTEIN 70, ANTIOXIDANT STATUS, BIOCHEMICAL AND HORMONAL PROFILES DURING SUMMER STRESS IN HALLIKAR CATTLE''*** submitted by **Mr. GURUBASAYYA P. KALMATH, I.D. No. DVHK 1230** in partial fulfillment of the requirements for the award of **DOCTOR OF PHILOSOPHY** in **VETERINARY PHYSIOLOGY** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of bonafide research work carried out by him during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

Place: Bangalore
Date: May, 2015

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Affectionately Dedicated

To

My Beloved Late Father

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LIST OF ABBREVIATIONS

%	Percentage
μIU/mL	Micro International Unit per milliliter
μmol	Micromoles
° C	Degree Celsius
ACTH	Adrenocorticotropic hormone
ALP	Alkaline Phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
cAMP	Cyclic adenosine monophosphate
CPM	Counts Per Minute
CRH	Corticotropin Releasing Hormone
ELISA	Enzyme linked immunosorbent assay
Fig.	Figure
g	Gram
g/dL	Gram per decilitre
GPx	Glutathione Peroxidase
GSH	Reduced glutathione
H_2O_2	Hydrogen peroxide
Hb	Hemoglobin
HDL	High Density Lipoprotein
HPA	Hypothalamo-Pituitary-Adrenal axis
HSP70	Heat Shock Protein 70
Ig	Immunoglobulin

IC	Immune Complexes
IL2	Interleukin 2
IU/L	International Units per litre
L	Litre
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
mg/dL	Milligram per decilitre
min	Minute
ml	Milliliter
ng/mL	Nanogram per milliliter
nmol/L	Nanomoles per liter
PBMC	Peripheral Blood Mononuclear Cells
PEG	Polyethylene Glycol
RH	Relative Humidity
RIA	Radioimmunoassay
ROM	Reactive Oxygen Metabolites
ROS	Reactive Oxygen Species
SE	Standard Error
SOD	Superoxide Dismutase
TBARS	Thiobarbituric acid reactive substance
T_{db}	Dry bulb temperature
TG-lipase	Triacylglycerol lipase
THI	Temperature Humidity Index
VLDL	Very Low Density Lipoprotein
µl	Micro liter

Introduction

I. INTRODUCTION

The livestock sector has contributed to the Gross Domestic Products (GDP) of the country to the extent of 4.1 per cent during the year 2012-13. This sector also provides a due share to the national food security. But, due to climatic changes, there is reduction in animal productivity which needs to be attended immediately to ensure optimum animal production.

Heat stress causes adverse effects on health, welfare and production of farm animals with huge economic losses to the livestock industry to the extent of about 60 per cent of the dairy farms around the world (Behl *et al*., 2010). This is attributed to increasing summer temperature worldwide as a result of climate change and it is predicted that the increasing trend will continue. The Intergovernmental Panel on Climate Change (IPCC), Geneva, predicts that by the year 2100, increase in the overall global temperature may be between 1.8 to 4.0 °C. Even if global surface temperature increases by 1.5 to 2.5 °C, approximately 20 to 30 per cent of the livestock and animal species are expected to be at risk of extinction (Aggarwal and Upadhyay, 2013).

In addition to the temperature, the intensity and duration of heat waves are also expected to increase dramatically. Therefore, the global warming and climate change are likely to become major threats to the sustainability of livestock production systems around the world in the days to come. India being the tropical country, extended periods of high ambient temperature and humidity will make the Indian livestock production system more prone for the challenges of climatic change.

Domestic animals are exposed to various kinds of stress such as physical, nutritional, chemical, psychological and thermal stress. Among these, thermal stress (heat stress) is the most important stress and the concern about environmental heat stress is increasing in recent years with the realization of impact of global warming on animal production.

Bovines, like other homeotherms, maintain their body temperature within narrow range and change in physiological and behavioural responses help in their thermoregulation process. Under thermoneutral environmental conditions most of the large domestic animals show optimum performance as they can successfully maintain equilibrium between heat production and heat loss.

Heat stress occurs when there is an imbalance between heat production and heat dissipation in animals. Heat stressed animal attempts to minimize the adverse effects by physiological mechanisms, such as an increased respiration rate, increased sweating and reduced metabolic rate. If the physiological adjustments fail, the wellbeing of the animal and the performance of the animal are affected.

Exposure of animals to thermal stress results in transcriptional activation and accumulation of a set of proteins called “Heat Shock Proteins” (HSPs). These proteins act as molecular chaperones in regulating the cellular homeostasis and folding and unfolding of damaged proteins during thermal injury (Kapila *et al*., 2013).

Various strategies are being followed for alleviating heat stress which include dietary modification to minimize diet-induced thermogenesis (low fibre and low protein),

increasing nutrient concentration in the feed to compensate for lower intake, managemental measures to reduce the heat load on the affected animals (shading / improving ventilation by using fans) or to enhance heat loss from their bodies (sprinklers / misters) and genetic selection for heat tolerant animals. Though the advances in managemental and nutritional strategies have alleviated some of the negative impacts of heat stress in cattle, the productivity continues to decline during the summer.

Indian zebu cattle *(Bos indicus)* are more heat tolerant animals compared to temperate cattle (*Bos taurus)* due to the presence of more number of sweat glands per square centimetre area of the skin. Hallikar is a *Bos indicus* draught breed of Mysore, a type of zebu cattle. It is a premier draught breed in India and they are often raised by families who are specialized in production of Hallikar animals for hundreds of years. They are found primarily in the southern region of Karnataka state, in the area surrounding Mysore and are known for their endurance to heat stress.

Defining the biological mechanisms of heat stress that jeopardize animal performance is critical and is essential for designing future mitigating strategies such as genetic, managemental, nutritional and pharmaceutical interventions to further improve the animal performance and to ensure constant supply of animal products for human consumption. It is also recognized that, the experimental trials on farmer owned animals in their own premises at rural conditions would give more realistic results than on-station experiments. Such experimental trials on the effects of summer stress in Hallikar cattle are lacking.

There is also lack of systematic study on cellular mechanisms associated with the thermotolerance ability and oxidative, biochemical and hormonal changes associated with the heat stress in native breed like Hallikar cattle. There is paucity of information on the effects of dietary supplementation of antioxidant agents like vitamin E and selenium on alleviation of heat stress in Hallikar cattle. Therefore, the present study was undertaken with the following objectives:

1. To study the effects of summer stress on the levels of Heat Shock Protein 70 (HSP70) and antioxidant enzyme activities in Hallikar cattle.
2. To elucidate the biochemical and hormonal profile in Hallikar cattle during summer stress.
3. To study the effects of supplementation of antioxidants (vitamin E and selenium) on HSP70, antioxidant enzyme activities, biochemical and hormonal profiles in Hallikar cattle exposed to summer stress.

Review of Literature

II. REVIEW OF LITERATURE

2.1 Summer stress and its impact on livestock

Economic losses associated with heat stress in livestock industry are mainly because of its negative effects on feed intake, daily weight gain, reproductive performance and embryonic development (Collier *et al*., 2006). Apart from the stresses due to calving, periparturient disease and metabolic demands, the environmental factors such as temperature and humidity may also disturb the delicate oxidant or antioxidant balance important for proper physiological functioning (Burke, 2007).

Heat stress is the state at which various mechanisms activate to maintain the thermal balance of the animal body, when exposed to intolerable elevated temperature. The primary environmental factors like ambient temperature, relative humidity and radiant energy affect the physiology of thermal regulation and maintenance of positive heat loss (Marai and Haeeb, 2010). Heat stress is particularly a severe problem in high yielding milch, dual purpose and drought cattle population (Behl *et al*., 2010).

Heat stress is one of the wide varieties of factors which cause oxidative stress *in vivo* (Sunil Kumar *et al*., 2010). Stress is broad term, generally used in negative connotation and is described as the cumulative detrimental effect of a variety of factors on the health and performance of the animals (Sunil Kumar *et al*., 2011).

Heat stress causes a significant negative economic impact for the dairy industry in arid and semi-arid regions of the world, so that heat abatement is an important issue for dairy producers (Avendano-Reyes *et al*., 2012). Under heat stress, a number of

physiological and behavioral responses occur that vary in intensity and duration in relation to the animal's genetic makeup and environmental factors. Environmental, nutritional, physical, social or physiological stressors are likely to affect the animal welfare and performance of the animals (Pankaj *et al*., 2013).

Heat stress phenomenon has become major issue in the current scenario of climate change and heat stress induced by hot environment reduces the productive efficiency in dairy animals. In order to improve the animal health and maximize their performance, the impact of heat stress must be minimized (Kapila *et al*., 2013).

Variation of climatic variables like temperature, humidity and radiation exert potential hazards and interfere with the growth and production of domestic animals. Higher environmental temperature along with high air humidity causes an additional discomfort to the animals and enhances the stress leading to depression of physiological and metabolic activities in the affected animals (Ganaie *et al.*, 2013b).

In Indian subcontinent, heat stress is the most important climatic stress which adversely affects the livestock and sometimes even threatens the survival of the animals (Sejian *et al*., 2013). Increased heat load, caused by a combination of air temperature, relative humidity, wind velocity and solar radiation, increases the body temperature and respiration rate, and can reduce the feed intake and milk production (Sreedhar *et al.*, 2013). Heat stress can be defined as the point where the animal cannot dissipate adequate quantity of heat to maintain body thermal balance. Various climatic factors like ambient temperature, relative humidity, radiation and wind also influence the degree of heat stress (Samal, 2013).

Thermal stress occurs due to single or combination of environmental factors when the effective temperature of the environment is higher than the animal's thermoneutral zone. Heat stress brings about a series of physiological, anatomical and behavioral changes leading to reduced productive performance in animals (Prava and Upadhyay, 2014).

The term stress is used to describe the influences outside of a body system, which can shift the internal mechanism away from their normal or resting state. Therefore, the term heat stress is used to describe the effects of increasing environmental temperature on various physiological systems (Baumgard *et al*., 2014).

Stress is the result of environmental forces continuously acting upon the animals and disturbances in homeostasis resulting in new adaptations that can be detrimental or beneficial to the animals (Al-Samawi *et al.*, 2014). Heat stress is a major stressor that occurs as a result of an imbalance between heat production within the body and its dissipation and it affects animals at cellular, molecular and ecological levels (Mehla *et al*., 2014). Heat stress is the most stressful abiotic stress to animals including farm animals. In tropical countries like India, summer is the most stressful season for animals because of higher temperatures beyond 42 °C (Kolli *et al*., 2014).

2.1.1 Assessment of severity of heat stress in animals

Animals' vulnerability to the weather is well established and their performance and survival are strongly influenced by weather. Consequently, weather is a constraint on efficient livestock production systems and the adverse effects of weather are further aggravated under warm to hot climatic conditions. Temperature humidity index (THI)

value of less than 70 is normal, a THI value of 70 to 78 is alarming, a THI value of 78 to 82 is considered as dangerous and THI value of 82 or above is considered as emergency (Du Preez *et al*., 1990).

THI is commonly used as an indicator of degree of climatic stress on animals where THI of 72 and below is considered as no heat stress (cool), 73 to 77 as mild heat stress (HS), 78 to 89 as moderate and above 90 as severe heat stress. In the study, on effect of heat stress in Omani and Australian Merino sheep, the THI of about 72 was observed during cooler month of December which increased up to 93 during the hotter month of July (Srikandakumar *et al*., 2003).

The effect of ambient heat on dairy cattle maintenance and milk production is well known and furthermore heavily influenced by relative humidity. A combination of the two variables (temperature-humidity index; THI) is a better predictor of whether or not cows are stressed (Baumgard *et al*., 2006). Trana *et al.* (2006) recorded significantly higher THI values (75.7 ± 2.8) during summer compared to spring season (56.2 ± 2.8). The THI at which cattle may maintain the stable body temperature is between 72 and 76 depending on the breed and air velocity (Gudev *et al*., 2007b). Marai *et al*. (2007) described the THI as a mean of estimating the severity of the heat stress using both ambient temperature and relative humidity. Further, they opined that THI <82 indicates the absence of the heat stress, THI of 82 to 84 indicates moderate heat stress, THI of 84 to 86 indicates severe heat stress and THI over 86 indicate extremely severe heat stress.

Singh and Upadhyay (2008) in their study on effect of thermal stress on physiological parameters and milk production used dry bulb and wet bulb temperature to

calculate temperature humidity index (THI) and observed an inverse relationship between THI and the milk yield. Further, they reported that each unit increase in THI decreased the milk yield by 0.101 L/day/animal when THI is between 72 to 82.

Magnitude of the heat stress is defined as the sum of forces external to the animal that act to displace body temperature from set point that is caused by the combined effects of dry bulb temperature (T_{db}), humidity, solar radiation, and wind speed (WS). Different indices are being used to estimate the degree of heat stress and the most common of these indices is the temperature-humidity index (THI), which utilizes dry bulb (T_{db}) and wet bulb temperature (T_{wb}) to estimate the magnitude of heat stress (Dikmen and Hansen, 2009).

In the study of Avendano-Reyes *et al*. (2010), THI that takes the account of both ambient temperature and relative humidity was used to quantify the degree of heat stress in animals. They recorded minimum and maximum temperature of 26 °C and 48 °C during summer months and the calculated THI ranged from 71 to 94. Aggarwal and Singh (2010) reported that the temperature humidity index was higher during hot-humid season (83.6) than during hot-dry season (80.7), indicating a higher level of heat stress during the hot-humid season.

Temperature Humidity Index that combines the simultaneous effects of both temperature and relative humidity is commonly used to quantify the degree of heat stress in animals (Beltrano *et al.*, 2010). Cincovic *et al.* (2011) studied the metabolic acclimation to heat stress in Holstein cows with different body condition scores and

recorded the maximum value of THI (75 to 80) that indicated moderate to high intensity of thermal stress.

Kim *et al.* (2012) calculated the mean daily THI utilizing the maximum temperature (°C) and average relative humidity (%) using the following equation:

$$THI = (0.8 \times T_{max}) + (\% \text{ average RH}/100) \times (T_{max} - 14.4) + 46.4$$

A temperature humidity index (THI) is commonly used to indicate the degree of heat stress in animals and high THI reduces the feed intake of animals, causing a negative energy balance and body weight losses in affected animals (Salama *et al*., 2013).

Ashatsham-ul Haq *et al*. (2013) opined that a THI value lower than 72 is ideal for tropical animal husbandry, a THI value of 72 to 78 causes mild stress, 78 to 88 causes moderate stress and THI above 88 causes extreme stress in cattle. They recorded overall mean THI of 74.06 ± 3.922 and 87.60 ± 4.32 in the morning and afternoon, respectively during the hottest months of the year.

The environmental conditions that induce heat stress can be calculated using the temperature humidity index.

$$THI = (\text{Dry bulb temperature } °C) + (0.36 \times \text{dew point temperature } °C) + 41.2$$

When the THI is more than 72, heat stress begins to occur in dairy cattle (Samal, 2013).

Zhang *et al*. (2014) studied the effect of different THI and chromium supplementation on lactating cows and opined that the temperature humidity index is

commonly used to indicate the degree of heat stress in dairy animals. Das *et al.* (2014) recorded the average temperature humidity indices of 82.75 and 91.18, and 83.40 and 86.40 in control group animal shed at 9 AM and 2 PM in hot dry and hot humid period, respectively.

The temperature humidity index is considered as a scale of measuring the heat stress on animals and THI above 72 exerts adverse effects on different breeds of cattle and buffaloes depending upon their adaptability to tropical climatic conditions (Singh *et al*., 2014). Study on thermoregulatory responses of Aardi goats exposed to heat stress revealed significantly higher ambient temperature and temperature humidity index during summer than in winter. However, the relative humidity was significantly lower during summer than winter (Al-Samawi *et al*., 2014).

2.2 Heat stress and Heat shock Protein 70 (HSP70)

Including domestic animals, the ability to adapt to the stress is of great importance, in all the organisms and at the cellular level eukaryotic cells respond to stresses by producing a set of proteins called heat shock or stress proteins (Guerriero and Raynes, 1990). Heat shock proteins (HSPs) family consists of many proteins which are classified based on their molecular weight and amino acid sequences as well as by structure and functions and cellular locations. Major HSPs in mammalian cells include HSP110, HSP100, HSP90, HSP70, HSP60, HSP40, HSP27, HSP10, and small HSP families (Feder and Hofmann, 1999). Heat shock proteins are involved in stabilizing the cells during exposure to increased temperature and heat shock for 1 h at 42 °C causes two to three fold increase in the intracellular concentration of the heat shock protein 70 in

Angus (*Bos taurus*, non-heat-tolerant), Brahman (*Bos indicus*, heat-tolerant) and Senepol (*Bos taurus*, heat-tolerant) breeds of cattle (Kamwanja *et al*., 1994). Thermotolerance is associated with HSP70 induction and heat conditioning resulting in an increased expression of HSP70 when animals were further challenged with more stressful stimuli (Wang and Edens, 1994). Increased HSP70 helps the cells to recover from previous stressors and provide them a transitory degree of protection (Li *et al*., 1995).

Heat shock proteins (HSPs) are highly conserved proteins present in both prokaryotic and eukaryotic cells and play important role in fundamental cellular processes. As the name indicates, HSPs are induced in the cells when exposed to sub-lethal heat shock. In 1962, for the first time, Ritossa reported an increased expression of proteins with molecular masses of 70 and 26 kDa in *Drosophila* salivary gland cells exposed to a temperature of 37 °C for 30 minutes. They are named as "heat shock proteins". Since then, a large number of proteins in addition to these, also collectively referred to as HSPs, have been discovered (Kiang and Tsokos, 1998).

Among the various HSPs, HSP70, with a molecular weight 70 kDa, is known to be the major molecular chaperone in all the cellular compartments and organs (Wang *et al*., 2003). Heat shock protein 70 is a potent survival protein that functions by inhibiting the death-associated permeability of lysosomes and the depletion of this protein triggers massive caspase-independent cell death (Nylandsted *et al*., 2004).

Significant increase in the plasma concentration of HSP70 was observed in Murrah buffalo heifers exposed to thermal stress. Mean plasma concentration of HSP70 in heat stressed buffaloes ranged from 1.76 to 2.25 ng /mg of total protein of the sample.

This increase in HSP70 indicates a lower thermotolerance for extreme heat exposure and is associated with higher expression of HSP70 (Patir and Upadhyay, 2007).

During summer, multiple cellular mechanisms operate to counteract the changes that occur due to heat stress. Cellular responses to heat stress include activation of heat shock transcription factor 1 (HSF1), increased expression of heat shock proteins (HSPs), decreased expression and synthesis of other proteins, increased glucose and amino acid oxidation and reduced fatty acid metabolism, activation of endocrine system and immune system activation via extracellular secretion of HSPs (Collier *et al*., 2008).

Patir and Upadhyay (2010) studied the temperature dependent induction and expression pattern of HSP70 in buffalo lymphocytes. The results of the study indicated that higher intensity and duration of the temperature exposure caused higher HSP70 induction in buffalo lymphocytes and it was attributed to the maintenance of cellular homeostasis. Heat shock protein 70 (HSP 70) bestows cytoprotection against various kinds of stresses. Among all the HSPs, HSP70 are ubiquitous, most abundant and temperature sensitive proteins. Goat heat shock protein 70-1 cDNA encoding HSP70 protein of 641 amino acid residues was found highly conserved among domestic animals. Among HSPs, heat shock protein 70 (HSP70) is an essential molecular chaperone of primary importance to all mammalian cells. HSP70 gene family in bovines consists of HSP70-1, HSP70-2, HSP70-3, and HSP70-4 gene (Gade *et al.*, 2010).

Exposure of cells to elevated temperature results in general inhibition of protein synthesis, defect in protein structure and function, cytoskeleton rearrangements resulting in the morphological changes, alteration in the cell membrane dynamics and fluidity and

decrease in cellular proliferation. These anomalies invoke extensive changes in the gene transcription and protein synthesis known as heat shock response (Behl *et al*., 2010). Cellular exposure to various conditions like hypothermia, oxidative stress, excess exercise, changes in pH, incorporation of new amino acids into proteins, viral infection, etc. rapidly enhance the intracellular concentration of HSP70 (Tkacova and Angelovicova, 2012).

HSP70 mRNA expression was significantly higher in goats during summer season as compared to winter season in tropical region. However, in the temperate region, HSP70 expression in peripheral blood mononuclear cells of goats did not vary during summer and winter seasons (Dangi *et al*., 2012).

Heat shock proteins (HSPs) are present in all the major cellular compartments and play an important role in protein homeostasis. They are so called because they were first observed in response to hyperthermia. Heat shock protein 70 (HSP70) is present in low concentrations as molecular chaperones in unstressed cell and is essential for protein synthesis, translocation and storage (Tkacova and Angelovicova, 2012). Perturbations of the cellular homeostasis such as heat stress causes preferential transcription of proteins called heat shock proteins that act as molecular chaperones (Charoensook *et al.*, 2012).

Without being part of the final protein structure, HSPs can acts as molecular chaperones by participating in the assembly of proteins. HSPs felicitate the cellular survival by reducing the accumulation of damaged or abnormal polypeptides within cells. They possess crucial role in intracellular transport, the maintenance of proteins in an inactive form and the prevention of protein degradation. Heat shock protein 60 is present

in mitochondria and helps in refolding of proteins and prevents aggregation of denatured proteins. HSP70 is present in cytosol and nucleus and helps in nascent protein folding and cytoprotection. HSP90 is present in cytosol, endoplasmic reticulum and nucleus. It assists in protein translocation and regulation of steroid hormone receptors (Gupta *et al*., 2013).

A study to determine the effect of chronic stress (housing, diet and climate) on extracellular heat shock protein 70 (HSP70) concentration in plasma in growing feedlot cattle showed a strong relationship between HSP70 concentration and ambient temperature, HSP70 concentration and photoperiod and no relationship with body temperature (Gaughan *et al*., 2013).

Various HSPs, that are members of molecular chaperone families, are known to be highly expressed under stressful conditions. These proteins provide protection to cells under stress and responsible for adaptation during and following stress (Kishore *et al*., 2013). Heat shock protein 70 is one of the most abundant and best characterized heat shock protein family that consist of highly conserved stress proteins, expressed in responses to stress, and play crucial roles in environmental stress tolerance and adaptation (Banerjee *et al*., 2013b).

Study on the effect of melatonin administration on the relative expression of HSP60, HSP70, HSP90 and ubiquitin genes in peripheral blood mononuclear cells showed significant up-regulation of all the genes during heat stress in both control and treatment group (Sharma *et al*., 2013). Aggarwal *et al*. (2013) studied the effect of supplementation of α-tocopherol acetate in periparturient crossbred cows and the results

obtained showed a significant reduction in the plasma HSP70 levels in cows supplemented with α-tocopherol when compared to their control counterpart.

Impact of vitamin E supplementation on heat shock protein 72 during seasonal stress in Murrah buffaloes was studied and the study concluded that the feeding of vitamin E lowers the levels of thermal stress markers like HSP72 mRNA expression in lymphocytes (Lallawmkimi *et al*., 2013). Heat stressed broiler birds showed significantly higher expression of HSP70 in the lymphocyte lysate as compared to the control group bird's lymphocyte (Roy *et al*., 2013a). HSP70 concentration was significantly higher in the tissue lysates of heat stressed broiler birds when compared to control counterparts (Roy *et al*., 2013b). HSPs are the molecular chaperones that promote the folding of nascent polypeptides and correct the mis-folding of denatured proteins (Gupta *et al*., 2013).

Banerjee *et al*. (2013b) observed significantly higher expression of HSP70 genes in cold-adapted goats during summer and in heat-adapted goats during winter. Further, they concluded that the expression pattern of HSP70 genes is species and breed specific, most likely due to variations in thermal tolerance and adaptation to different climatic conditions.

Studies on expression of HSP70 in dermal fibroblasts to thermal stress in Tharparkar and Karan-Fries cattle indicated that all HSP70 genes were up-regulated at different temperatures in both the breeds and the relative expression of inducible HSP70 were higher in Karan-Fries than Tharparkar. The study suggested that zebu cattle

(Tharparkar) dermal fibroblasts are more adapted to tropical climatic conditions than cross breed cattle (Karan-Fries) (Singh *et al*., 2014).

Heat load in both Sahiwal and Karan-Fries breeds of cattle did not enhance the expression of HSP72 in peripheral blood mononuclear cells (PBMC) and both breeds showed similar response to the short term thermal exposures in expressing the HSP72 (Prava and Upadhyay, 2014). Zhang *et al*. (2014) studied the effect of temperature humidity index and chromium supplementation on antioxidant capacity and heat shock protein 72 of lactating Holstein cows and found that there was no difference in the expression of HSP72 between the cows with or without chromium supplementation during high temperature humidity index period.

Deb *et al*. (2014) studied the effect of heat stress on expression profile of HSP90 in peripheral blood mononuclear cells among Sahiwal and Frieswal breeds of cattle and found that Sahiwal express higher levels of HSP90 than Frieswal to regulate their body temperature and increase the cellular survivability under heat stressed conditions. Differential expression of HSPs in animals during thermal stress may be responsible for better stress tolerance of the tropical breeds of cattle compared to European breeds (Deb *et al.*, 2014). HSP70 plays vital role in cryoprotection and is frequently used as a biomarker of cellular stress. Expression levels of HSP70 are indicative of magnitude and duration of the thermal stress (Rhoads *et al*., 2013b).

2.3 Free radicals and oxidative stress

Toxicity of the oxygen, in spite of its requirement for all the living aerobic organisms is termed as “oxygen paradox”. The term "reactive oxygen metabolites"

(ROMs) has been applied to oxygen-centered free radicals and their metabolites. Some ROMs are produced endogenously by normal metabolic processes, but their amount increase markedly by exogenous factors, including solar radiation, fungal toxins, and pesticides (Miller and Brzezinska-Slebodzinska, 1993).

Enhanced production of oxygen free radicals as a result of elevated temperature causes deleterious effects. Reactive oxygen species compromise the cellular function by removing electrons from a variety of molecules. Superoxide anion (O_2^-) is the major free radical produced in mitochondria from electron transport chain (Trout *et al*., 1998). Reactive oxygen species (ROS) are an unavoidable part of normal cellular metabolism. Imbalance between ROS generation and antioxidants can lead to oxidative stress (Anita *et al*., 2003). Plasma antioxidant vitamins and minerals such as vitamin C, E, folic acid, and zinc levels decline during oxidative stress resulting in oxidative damage to the body cells (Gursu *et al.*, 2004).

Reactive oxygen metabolites, generated during normal cellular metabolism, are involved in several physiological functions. Production of ROMs in excess quantities than they can be removed safely by the antioxidant mechanisms, may lead to a state called oxidative stress (Trana *et al*., 2006). Interaction of prooxidants and antioxidants in living systems determines biological redox state. Shifts in redox balance have important implications for animal health and function. Excessive prooxidant species or inadequate antioxidant defenses lead to oxidative stress, a condition that is practically interpreted with reference to resultant functional impairment in the animal body (Burke, 2007).

Oxidative stress is the result of an excessive exposure to oxidants and inadequate availability of antioxidants, or a combination of both. Oxidative stress leads to peroxidative damage of the lipids as indicated by increase in thiobarbituric acid reactive substances (TBARS) levels (Aggarwal *et al*., 2008). Molecular oxygen is required as an electron acceptor for the efficient production of energy in all living aerobic organisms and free radicals are formed as a normal end product of cellular metabolism. Free radicals are the molecules that have at least a single unpaired electron in the outer orbit and can promote electron transfer through oxidation and reduction reactions (Sordillo and Aitken, 2009).

Oxidative stress occurs when the antioxidant defense systems is overwhelmed by an increased oxidant burden or reduced antioxidant supply. The reactive nitrogen species (RNS) have been identified as a sub-group of oxidants derived from nitric oxide (Hala *et al*., 2009). Reactive oxygen species (ROS), the major molecules causing oxidative stress, are constantly generated *in vivo* as an integral part of the metabolism. Despite acting as first line of defense in combating the infection, ROS may cause oxidative stress when their level exceeds the threshold value (Sunil Kumar *et al*., 2010 and Sunil Kumar *et al.*, 2011). Oxidative stress results when reactive oxygen metabolites (ROM) generation exceeds the capacity of antioxidant system of the cell, tissue, or body. Major ROMs produced during heat stress are superoxide, hydrogen peroxide, hydroxyl radical and fatty acid radicals. These ROMs react with various enzymes, cell membranes and deoxyribonucleic acid (DNA) causing cell damage or cell death (Tahmasbi *et al*., 2012).

Low level of ROS is essential for all the cells in the body to provide energy for vital functions. During aerobic metabolism, about 95 to 98 per cent of consumed oxygen is reduced to water and the remaining fraction may be converted into oxidative by-products like ROS that contribute to the oxidative stress (Ganaie *et al*., 2013b). Increased oxygen demand in the body augments the production of oxygen derived reactants, collectively termed as reactive oxygen species. When ROS are produced faster than they can safely be neutralized by body antioxidant system, oxidative stress results (Maurya *et al*., 2013). Exposure to the environmental variations can produce oxidative stress due to cytotoxic and mutagenic activity as well as aberrant changes to cell cycle progression and replication (Kataria *et al*., 2013). The role of free radicals is gaining increasing worldwide attention since so many physiological and patho-physiological phenomena are related to redox status cell modification (Nisar *et al*., 2013).

Disturbance in the balance of prooxidants and antioxidants, in favor of prooxidants causes potential damage to the cells and organs in the body of the affected animal is known as oxidative stress (Bhat *et al*., 2008 and Nisar *et al*., 2013). Oxidative stress results from increased production of free radicals and reactive oxygen species, and / or a decrease in the antioxidant defense leading to damage to the biological macromolecules and disruption of normal metabolism and physiology (Bernabucci *et al*., 2002 and Chigerwe *et al*., 2013).

Oxidative stress is a disruption of redox signaling leading to damage of macromolecules such as lipids, proteins, DNA, and disruption of normal metabolism and physiology leading to loss of cell function. Oxidative stress in veterinary medicine and

particularly in ruminant health is a relatively young field of research (Celi and Chauhan, 2013).

Different types of physical and chemical stressors such as heat, cold, transportation, diseases and exposure to various environmental pollutants induce production of reactive oxygen species (ROS) that results in oxidative stress leading to peroxidation of bio-molecules such as lipids, proteins and nucleic acids (Devasena and Adilaxmamma, 2014).

2.3.1 Antioxidants against oxidative stress

The components of the antioxidant system in the body have been classified as preventive or chain breaking. Preventive system includes both metal-binding macromolecules and antioxidant enzymes. Metal-binding macromolecules like transferrin, ceruloplasmin, and albumin will take care of ROM reactions in extracellular fluid. Inside the cells, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase cause removal of O_2^- and H_2O_2. Chain breaking antioxidants act after the initiation of the chain reaction and this class includes lipid soluble vitamin E, ubiquinone, and β-carotene and water soluble ascorbate, GSH, and urate (Miller and Brzezinska-Slebodzinska, 1993).

Depletion of any component of the antioxidant systems enhances the vulnerability of various tissues and cellular components to reactive oxygen species, while tissues seem to increase their antioxidant defences under chronic activation (Avellini *et al*., 1999).

The activity of most of the antioxidant enzymes in the body is influenced by nutrition and heat stress. In dairy cows, deficiencies of dietary antioxidants can result in oxidative stress (Trana *et al.,* 2006).

Animal antioxidants are diverse and can be either synthesized in the body or derived from the diet, and are localized transiently throughout tissues and different cell types (Spears and Weiss, 2008). Antioxidant is any substance that delays, prevents or removes oxidative damage to target molecules. ROS production during oxygen metabolism necessitates activation of antioxidant defense system that effectively traps reactive intermediates before they cause damage to macromolecules. Selenium-dependent antioxidant enzymes, however, are the most widely studied systems with respect to dairy cattle health and well-being (Sordillo and Aitken, 2009).

To protect the body against the menace of heat stress induced free radicals, it employs two types of antioxidants namely enzymatic and nonenzymatic antioxidants. Enzymatic antioxidants include SOD, GPx and CAT. Nonenzymatic antioxidants include vitamins like vitamin C, E and A, proteins like albumin, transferrin and reduced glutathione (GSH) (Sunil Kumar *et al*., 2010).

Excessive production of free radicals and concomitant damage at cellular and tissue levels are controlled by cellular antioxidant defense systems (Maurya *et al*., 2013). Antioxidants are the nutrients (vitamin A, E and C) that significantly delays or inhibits oxidation process in very low concentrations and hence are essential to cleanse the cells (Ganaie *et al.,* 2013b).

Animals maintain a complex system of multiple types of antioxidants such as glutathione, vitamin C and E as well as enzymes such as catalase, superoxide dismutase (Devasena and Adilaxmamma, 2014). Glutathione reductase, superoxide dismutase and catalase act as antioxidants in the body and help in catalyzing the respective free radicals or oxidants (Yatoo *et al*., 2014).

2.3.1.1 Amelioration of oxidative stress by nutritional supplementation

Vitamin E and selenium are the two components which contribute to the antioxidant potential of the plasma and tissue. Dietary modulation with inclusion of vitamin E and selenium is used to correct the oxidant-antioxidant imbalance (Doni *et al*., 1984). Ealy *et al*. (1994) used two different dose combinations to supplement vitamin E and selenium to Holstein cows exposed to heat stress during the months of July and August, *viz*., 675 IU of vitamin E with 7 mg/day of selenium per cow and 1000 IU of vitamin E with 7.125 mg/day of selenium and none of the combinations were found effective in alleviating the heat stress during the summer season.

Supplementation of vitamin E and selenium to the postpartum anoestrus buffaloes decreased the levels of lipid peroxidation and increased the plasma levels of vitamin E and β-carotene resulting in improved reproductive efficiency (Anita *et al*., 2003). Antioxidants like vitamin C, vitamin E, vitamin A and zinc provide effective protection to the body against the oxidative damage and therefore such nutrients can be included in the diet to prevent the negative effects of the environmental stress. Plasma antioxidant vitamins and minerals such as vitamin C, E, folic acid and zinc levels decline during oxidative stress resulting in oxidative damage to the body cells and their supplementation

will help the stressed animals to overcome the negative effect of the stress (Gursu *et al.*, 2004).

Trace minerals are an important source of dietary derived antioxidants and are known to play an important role in mitigating the oxidative stress (Spears and Weiss, 2008). Role of antioxidants in health and disease was studied extensively in both human and animal medicine (Sordillo and Aitken, 2009). Levels of selenium and vitamin E above the generally accepted requirements enhance the immune response in several species (Hala *et al*., 2009). Sivakumar *et al*. (2010) supplemented the Black Bengal goats with vitamin E @ 250 mg/animal/day as alpha tocopherol with selenium @ 0.1 mg/animal/day as sodium selenite and showed that the combination exerts ameliorative effect during heat stress.

Antioxidant nutrient supplementation especially vitamin C, A and E, zinc and chromium can be effectively used to attenuate the negative effects of environmental stress in poultry, smaller ruminants and rats (Sunil Kumar *et al*., 2010). Vitamins and minerals are vital nutrients that are involved in both metabolic and physiological processes, which are critical for human and animal health and animal food production (Kanchana and Jeyanthi, 2010). Certain nutrients act as antioxidants or components of antioxidant enzymes and have a direct effect on oxidative stress. Several trace minerals (as part of enzymes such as Se in structure of glutathione peroxidases) and some vitamins (such as vitamin E and vitamin C) are integral components of the antioxidant system (Tahmasbi *et al*., 2012).

Lipotropic factors, such as methionine, choline, and vitamin E are used to treat or prevent fatty liver syndrome and fatty liver hemorrhagic syndrome in poultry. Dietary choline, vitamin B_{12} and vitamin E decrease the hepatic triacylglycerol accumulation in laying hens due to its lipotropic effect (Choi *et al*., 2012).

During thermal stress, there is a significant reduction in the feed intake by livestock. Hence, ensuring the provision of nutrient dense diet during heat stress will help to minimize production losses due to high temperature. Feeding antioxidants (vitamin A, C, E, selenium and zinc) reduces the heat stress and optimize the feed intake (Pankaj *et al*., 2013). Antioxidants most importantly, vitamin E and selenium, play a major role in protecting the cells from ROS by reducing free radicals and preventing lipid peroxidation (Habibian *et al*., 2014).

Vitamin E (α-tocopherol), an antioxidant that prevent the oxidative damage, is a potent quencher of free radicals and contributes significantly to conservation of NADPH reducing equivalents by compensating for the glutathione S-transferase chain breakers (Lallawmkimi *et al*., 2013). Use of mineral and vitamins as potential alternatives for enhancing the livestock productivity has been increasing in the recent past and supplementation of the diet with antioxidants has been indicated to improve the health and production performances of farm animals by reversing the negative effects of oxidative stress (Celi and Chauhan, 2013).

Selenium and vitamin E deficiency could compromise the animal immune system and result in a decline in resistance of animals to infections. But, selenium and vitamin E

supplementation enhanced the immune response of lambs challenged with a viral pathogen (Sayed-Ahmed *et al*., 2015).

2.3.1.2 Selenium as an antioxidant

The health benefits of selenium (Se) are mediated by Se-containing antioxidant enzymes that prevent oxidative stress by reducing ROS to less reactive molecules, thus restoring an appropriate balance of reduced and oxidized molecules within cells (Sordillo, 2005). The importance of Se in the diet of dairy cattle is especially well documented based on its ability to reduce the incidence and severity of disease during times of heightened oxidative stress (Spears and Weiss, 2008).

Diets for ruminant livestock are almost exclusively of plant origin and the selenium concentration within plants can be extremely variable. Consequently, the concentration of dietary selenium can be deficient and dietary supplementation is required. Selenium supplements are in two principle forms, inorganic mineral salts like sodium selenite (Na_2SeO_3) or selenate (Na_2SeO_4) and inorganic form such as Se enriched yeast (Juniper *et al*., 2009).

Selenium is a vital constituent of the biologically important enzyme glutathione peroxidase (GSH-Px) which reduces the peroxides in cells thus preventing oxidative injury to cells (Khanal and Knight, 2010). Selenium plays immunomodulatory and anti-inflammatory roles in the animal. Selenium up-regulates expression of interleukin-2 (IL-2) receptor and increases their response of T and B lymphocytes to IL-2 (Calamari *et al*., 2010). Natural and synthetic antioxidants in the feed as well as optimal levels of

minerals, principally selenium, help to maintain efficient levels of endogenous antioxidants in tissue/s (Calamari *et al*., 2011).

Various minerals being integral part of the metalloenzymes like glutathione peroxidase (Se), catalase (Fe) and superoxide dismutase (Cu, Zn and Mn) are also critical in protecting the internal cellular constituents from oxidative damage (Maurya *et al*., 2012).

Selenium, an essential part of the antioxidant defense system, plays an important role in the growth and health of humans and livestock through its participation in several important enzymes and enzyme reactions (Alhidary *et al*., 2012). Selenium as an essential component of selenocysteine-containing protein is involved in most aspects of cell biochemistry and function (Tahmasbi *et al*., 2012).

Trace minerals are essential for health and immunity. As they are the integral part of the metalloenzymes and metalloproteins, they are involved in oxidation and reduction reactions and their deficiency predisposes the animal cells to oxidative stress (Yatoo *et al*., 2013). Selenium is an essential trace mineral in dairy cattle that is required to maintain normal physiological functions and provides a significant dietary source of antioxidant defenses (Sordillo, 2013).

Selenium is an essential element in the diet of the animals and is important in host antioxidant defense and immune function. A severe selenium deficiency can lead to sudden death from white muscle disease and a deficiency can results in ill thriftiness,

reproductive losses, reduced reproductive efficiency, wool yields and reduced growth rate in young lambs throughout the growing periods (Celi and Chauhan, 2013).

Selenium exists in four oxidation states and as a result it plays various important roles in biochemical transformations. In animals, the highest concentration of Se is observed in muscles, liver, blood plasma, erythrocytes and kidneys. Selenium is an integral part of proteins called selenoproteins (act as biocatalysts); biochemical functions of 30 of them are established and most of the selenoproteins are connected to DNA (Krzyzewski *et al*., 2013).

Selenium is an important trace mineral, acting in synergism with vitamin E which inhibits the oxidation of membrane polyunsaturated fatty acids and DNA by oxygen radicals produced during aerobic metabolism (Moeini and Jalilian, 2014). Selenium is an essential component of several major metabolic pathways, including antioxidant defense system, immune function and thyroid hormone metabolism (Sethy *et al*., 2014).

2.3.1.3 Vitamin E as an antioxidant

Vitamin E, a naturally occurring antioxidant, is found in abundance in the environment. It is an essential fat-soluble vitamin and is a generic name describing bioactivities of two of its derivatives, tocopherol and tocotrienol. The biological activity of vitamin E is believed to be due to its antioxidant action to inhibit lipid peroxidation in biological membranes by scavenging the peroxyl chain reaction. Vitamin E is known to have scavenger effect on reactive oxygen species and a stabilizing effect on damaged cell membrane (Dauqan *et al*., 2012).

Vitamin E acts as the first line of defense against lipid peroxidation, protecting polyunsaturated fatty acids in cell membranes through its free-radical-quenching activity in bio-membranes at an early stage of free-radical attack (Packer, 1991). Vitamin E is the most important lipid-soluble chain breaking antioxidant in the tissues, red cells and plasma which protect the cellular components against the peroxidative damage via the free radical scavenging mechanism or as a constituent of the membranes (Ibrahim *et al.,* 1997).

Tissue defense mechanisms against free-radical damage generally include vitamin C, vitamin E, and β-carotene as the major vitamin antioxidant sources. Vitamin E, the primary lipid-soluble, low-molecular-weight non-enzymatic antioxidant is important for the body's defense against oxidative stress (Ibrahim *et al*., 1997). Vitamin E, β-carotene and vitamin C have the ability to protect the biological membranes and tissues from oxygen toxicity and free radical attack, by rapidly scavenging the reactive oxygen species (Anita *et al*., 2003).

Vitamin E prevents oxidative damage to the membrane lipids by destroying the hydroperoxide formation and acting along with the selenium. It helps in maintaining the membrane integrity and reduces oxidative stress through inhibition and destruction of endogenous peroxides (Dimri *et al.*, 2010). Antioxidants such as vitamin C and E are free radical scavengers, which stimulate the body defense system to protect the body against excessively produced free radicals during heat stress and stabilize the health status of the animal (Sivakumar *et al.,* 2010).

Vitamin E plays an important role as a chain-breaking lipid antioxidant and free radical scavenger in the membranes of cells and sub-cellular organs (Habibian *et al*., 2014). Chandra *et al*. (2013a) supplemented the vitamin E @ of 1000 IU/day/cow along with zinc @ 60 ppm/day/cow during peripartum period to Sahiwal cows and observed significant increase in the milk production, by reducing negative energy balance.

2.3.4 Determination of oxidative stress

Erythrocytes are an appropriate and sensitive model to study the oxidative status of the transition dairy cows exposed to hot environments. Erythrocyte oxidative markers significantly increased in summer stressed dairy cows when compared to cows exposed to spring season and there was no significant change in the plasma oxidative markers (Bernabucci *et al*., 2002).

Oxidative stress can be monitored with several biomarkers and several methods have been developed to assess the total antioxidant capacity in view of the difficulty of measuring each antioxidant component separately and their interaction in the serum (Castillo *et al*., 2005).

Estimating the activities of enzymatic antioxidants, such as SOD and GPx is a mean of evaluating oxidative stress (Sathya *et al*., 2007). The activities of antioxidant enzymes and lipid peroxidation alter significantly during oxidative stress therefore they can be used as markers of oxidative stress (Sunil Kumar *et al*., 2010).

Compromised antioxidant defense and increased peroxidation products in blood are indicative for the oxidative stress. Measurement of lipid peroxidation in terms of

malondialdehyde, reduced glutathione, superoxide dismutase and catalase activities in biological samples are widely used to assess the oxidative stress (Singh *et al*., 2011).

Oxidative stress is not a classical disease to exhibit a specific clinical picture, therefore determination of products of macromolecular peroxidative damage and antioxidant substances like glutathione and enzymes like SOD, GPx and catalase are useful markers for determination of oxidative stress and antioxidant status, respectively (Sharma *et al*., 2011).

The nature and severity of oxidative stress can be determined by direct or indirect measures of oxidant and antioxidant activity. Several methods have been developed to measure biomarkers of peroxidation damage, the concentration of antioxidants and/or the activity of antioxidant enzymes (Celi, 2011). Measurement of lipid peroxidation in terms of malondialdehyde (MDA), reduced glutathione (GSH) level, super oxide dismutase (SOD) and catalase (CAT) activities in biological samples are widely used to determine the state of oxidative stress (Singh *et al*., 2011).

High polyunsaturated fatty acid content of erythrocyte membrane and the continuous exposure to high concentration of oxygen and iron in hemoglobin make the erythrocytes very sensitive to oxidative injury and make them an appropriate model to study oxidative stress (Kumar *et al.*, 2011). Plasma and/or erythrocytes biomarkers are being used to evaluate oxidative stress in various animal species. Biomarkers used to assess oxidative stress in adult cattle include plasma or erythrocyte glutathione peroxidase, reactive oxygen metabolites, intracellular and plasma thiols, superoxide dismutase and thiobarbituric acid reactive substances (Chigerwe *et al.*, 2013).

The antioxidant enzymes like glutathione reductase (GSH), (SOD and CAT along with the oxidant like malondialdehyde constitute oxidative stress indices. Estimation of oxidative stress indices in the body will enable to predict the level of oxidative stress in animal (Yatoo *et al*., 2014).

2.3.5 Erythrocyte catalase activity as an indicator of heat stress

Catalase is a heme-containing enzyme that catalyzes the dismutation of hydrogen peroxide into water and oxygen. Superoxide dismutase in conjugation with catalase and glutathione peroxidase scavenges both intracellular and extracellular superoxide radicals and prevents lipid peroxidation (Aggarwal and Prabhakaran, 2005).

Study on oxidative stress and antioxidant status in crossbred periparturient cows showed increase in the erythrocyte catalase activity in both control and DL-α-tocopherol treated animals towards calving and the activity gradually declined after parturition. Erythrocyte catalase activity was significantly higher in control group of animals compared to their treatment counterparts fed with DL-α-tocopherol (Chandra and Aggarwal, 2009).

Kumar *et al*. (2007) reported significantly higher erythrocyte catalase activity in heat exposed cattle and buffaloes compared to that of animals kept under natural climate. Sunil Kumar *et al*. (2011) reported significant decrease in activity of serum catalase upon exposure to the hot dry heat stress in both control and experimental group of buffaloes. The experimental group animals showed significantly less enzyme activity as compared to the control animals.

Kumar *et al*. (2011) studied the effect of dietary supplementation of antioxidants during heat stress in buffaloes wherein they have observed significant increase in the erythrocyte catalase activity in buffaloes of both control and supplemented groups when they are exposed to heat stress during hot dry and hot humid conditions. Further, the control group had significantly higher catalase activity compared to the group supplemented with sodium bicarbonate, potassium carbonate and ascorbic acid during both hot dry and hot humid conditions.

Ganaie *et al.* (2013a) conducted a study on effect of vitamin C on oxidative stress and immune status in pregnant Murrah buffaloes during thermal stress and reported significant increase in the activity of plasma catalase with the advancement of pregnancy from day - 45 to day 0 (day of parturition) in both control and supplemented groups. However, the increase in their activity was significantly higher in the control group compared to the vitamin C supplemented group indicating oxidative stress imposed by thermal stress in control group and restoration of normalcy by feeding of vitamin C in supplemented group.

Lallawmkimi *et al.* (2013) studied the impact of vitamin E supplementation in Murrah buffaloes and reported a significantly higher catalase in control group animals as compared to the vitamin E supplemented animals. The significant decline in the absolute erythrocyte catalase activity in vitamin E fed group indicate the beneficial effect in reducing the heat stress in buffaloes. Study of Yatoo *et al*. (2014) on seasonal changes of blood antioxidants in cattle and buffaloes revealed significant increase in the activities of

erythrocyte catalase during summer and winter than spring season indicating the oxidative stress due to too high and too low temperature.

2.3.6 Erythrocyte Superoxide dismutase (SOD) activity

Bernabucci *et al.* (2002) observed significant increase in the erythrocyte super oxide dismutase activity in Holstein cows during summer season when compared to the spring season. Activity of SOD progressively increased in the last 3 weeks of pregnancy, and reached the maximum, 4 days before parturition. The SOD activity rapidly declined after calving to reach the levels registered before calving (Bernabucci *et al*., 2005).

Trana *et al*. (2006) reported that the super oxide dismutase activities were significantly influenced by the season and significantly higher activity was observed in the hemolysate of dairy Red Syrian goats exposed to hot season during the mid lactations. The increase in the antioxidant enzyme concentration during summer over spring indicated moderate oxidative stress in lactating goats.

Wistar albino rats exposed to heat stress showed a significantly decreased erythrocyte SOD activities (2145 ± 635.38 U/g Hb) compared to the rats exposed to cold stress (3172.02 ± 863.2 U/g Hb). Increase in cortisol levels during hot season was responsible for enhanced oxidative stress in heat stressed group and was reflected by reduced erythrocyte SOD activity (Bhat *et al*., 2008).

Megahed *et al*. (2008) studied the influence of heat stress on cortisol and oxidant-antioxidant balance and reported a significant decrease in the serum antioxidant SOD in control group she buffaloes during summer as compared to winter. During summer

season the animals receiving antioxidant treatment showed significantly higher serum SOD activity.

Chandra and Aggarwal (2009) in their study on oxidative stress and antioxidant status in crossbred periparturient cows, recorded increase in the erythrocyte SOD activities towards calving and after parturition in both control and treatment animals. But, significantly lower SOD activities were reported in animals supplemented with DL-α-tocopherol compared to control group animals on all the observation days indicating the beneficial effect of dietary supplementation of DL-α-tocopherol.

Superoxide dismutase activity gradually decreased with the progression of pregnancy in control animals and though the same trend was observed in treatment group animals (Group II and Group III), the activity of SOD in Group III was significantly higher than the corresponding values in Group I and Group II on day 240, 280 and 290 of pregnancy. The study indicated that the supplementation of vitamin E and selenium has beneficial effect in terms of increasing the antioxidant enzyme (SOD) concentration in the body (Dimri *et al*., 2010).

SOD catalyzes the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen. Hydrogen peroxide is further degraded to water by other antioxidant enzymes, such as glutathione peroxidase and catalase. In mammalian cells there are three types of SODs: cytosolic superoxide dismutase, mitochondrial manganese superoxide dismuatase and extracellular superoxide dismutase (Sharma *et al*., 2011).

Sunil Kumar *et al*. (2011) studied the effect of supplementation of electrolytes, ascorbic acid and zinc on heat stress in buffaloes and reported a significant decrease in serum super oxide dismutase (SOD) activity in both experimental and control group buffaloes on exposure to hot dry and hot humid conditions. Further, the serum SOD activity is significantly reduced in experimental animals compared to their control counterpart.

Kumar *et al*. (2011) observed significant increase in erythrocytes SOD activity in control and supplemented (sodium bicarbonate and vitamin C) groups of buffaloes upon exposure to heat stress during hot dry and hot humid conditions. However, supplemented group showed significantly lower erythrocyte SOD activity than the control group animals indicating the reduced heat stress in antioxidant supplemented buffaloes. Sakatani *et al*. (2012) reported a significant decline in SOD activity in peripheral blood cells of Japanese Black cows during summer season.

Significant increase in the activity of plasma superoxide dismutase was observed in pregnant Murrah buffaloes with advancement of pregnancy in both control and supplemented group. But, the increase was significantly higher in control group compared to the vitamin C supplemented group indicating the beneficial effect of supplementing the vitamin C in reducing the oxidative stress in pregnant buffaloes (Ganaie *et al.*, 2013a).

A study on plasma oxidative status in neonatal dairy calves during summer and fall season reported significantly higher mean activities of plasma superoxide dismutase

in calves born and raised in summer, compared to calves born in fall indicating that the calves born and raised in summer are exposed oxidative stress (Chigerwe *et al*., 2013).

Deka *et al*. (2013) recorded the highest serum SOD activity (7933 ± 41.82 U/L) in weaning piglets supplemented with higher levels of zinc and copper. But the group supplemented with lowest zinc and chromium showed lowest serum SOD activity (5712 ± 19.98 U/L).

Zhang *et al*. (2014) reported a significant elevation in the activity of serum SOD during both moderate and high THI period compared to low THI period. They also showed that the chromium supplementation did not affect the activity of the serum SOD in Holstein cows.

Maurya *et al*. (2013) studied the effect of supplementation of vitamin E and zinc on transition period induced stress in Karan-Fries cows and they reported the mean erythrocyte SOD activities of 2793.89 ± 138.40 units/min/g Hb and 2710.42 ± 205.29 units/min/g Hb in control and treatment cows, respectively, on 60 days before the parturition. Mean erythrocyte SOD activities in control and treatment cows were 4658.73 ± 105.38 units/min/g Hb and 3858.63 ± 93.49 units/min/g Hb, respectively, on the day of parturition. Further, they observed that the increase in the activity of erythrocyte SOD in control and treatment group was 66.75 % and 42.36 % from 60 days before parturition to the day of calving. From their study they concluded that the increase in the activity was more significant in control group animals than the treatment animals.

Study of Lallawmkimi *et al.* (2013) revealed significantly higher erythrocyte SOD activity in control group animals under natural environmental conditions. But, significant decline in the absolute erythrocyte SOD activity in vitamin E fed group of growing, heifers and lactating buffaloes, indicating the beneficial effect of vitamin E supplementation at different physiological stages in buffaloes.

Yatoo *et al*. (2014) reported significantly higher activity of erythrocyte SOD in lactating and non-lactating cattle during summer and winter season than spring season and the same was attributed to the better adaptability of the cattle to optimum temperature than too hot or too cold temperature under tropical climate.

2.3.7 Erythrocyte Glutathione peroxidase (GPx) activity

Doni *et al*. (1984) reported a significant increase in the erythrocyte GPx activity in selenium supplemented rats than in control group rats. However, no significant increase in GPx activity was observed in vitamin E fed rats compared to control group animals.

Significantly higher erythrocyte GPx activity was observed in the Holstein cows during the summer season as compared to the spring season. However, the plasma GPx activity did not differ significantly between the summer season and spring season. Increase in activity of erythrocyte GPx during summer suggests that erythrocytes rather than plasma can be used as sensitive indicator of oxidative stress during hot environment (Bernabucci *et al*., 2002).

Study of Bernabucci *et al*. (2005) revealed a significant decline in the erythrocyte GPx activity between −4 and +11 days around calving. Glutathione peroxidase activity of plasma increased from the week before calving until first 30 days of the lactation. Plasma GPx showed significantly higher activity after calving (1.60 U/mL) compared to the activity before calving (0.84 U/mL).

Study on effect of season and nutrition on oxidative status in Red Syrian goats showed a significant increase in the activity of erythrocyte glutathione peroxidase during summer season compared to spring season (Trana *et al*., 2006). Burke (2007) reported significantly ($P<0.01$) lower activity (53.6 mU/mg) of GSH-Px in peripheral blood mononuclear cells (PBMC) of cattle during the month of July (highest THI - 80.1) compared to activity (83.2 mU/mg) of June (THI - 74.6) and activity (72.9 mU/mg) during August (THI- 77.3) months.

Bhat *et al*. (2008) reported a significantly decreased erythrocyte GPx activity in rats exposed to elevated ambient temperature of hot season (70.94 ± 10.79 U/g Hb) as compared to the rats exposed to cold season (80.12 ± 8.13 U/g Hb).

Study of Calamari *et al*. (2011) revealed significant positive influence of THI on the plasma GPx-3 activity (U/l). Further, supplementation with selenium significantly increased the plasma GPx-3 activity in Italian Friesian cattle indicating the improvement in the preventive antioxidant systems in the animal body during heat stress.

Glutathione peroxidase activity (units/ml PCV) in the blood plasma of Holstein cows during prepartum period was significantly higher in glutamine (100g/d)

supplemented group (57.44 and 45.87) as against the control group (47.94 and 41.72) animals at 7 days and 14 days before parturition, respectively. The study indicated that the supplementation of diets with glutamine on the close up period could enhance the plasma glutathione peroxides activity (Tanha *et al*., 2011).

Glutathione peroxidase is a selenium dependent antioxidant enzyme (Ganaie *et al*., 2013b) that catalyzes the reduction of organic hydroperoxides, lipid peroxides, and hydrogen peroxide, using glutathione as the reducing agent, thereby also protecting cells from oxidative damage resulting from normal oxidative metabolism. There are four known GPx that contain selenocysteine at the active site (Sharma *et al.*, 2011). Significantly lower levels of glutathione peroxidase were observed in peripheral blood cells of Japanese Black cows during summer season (Sakatani *et al*., 2012).

Deka *et al*. (2013), in their study on the effect of supplementation of zinc and copper on expression of thyroid hormones and anti-stress enzymes reported a highest serum GPx activities (350.05 ± 4.07 U/L) in the group of animals supplemented with higher levels of zinc and copper, whereas, the group that received lowest supplementation of zinc and chromium showed lowest serum glutathione peroxidase activity (310.99 ± 9.07 U/L).

Chigerwe *et al*. (2013) compared the plasma oxidative status of neonatal dairy calves during summer and fall season and found higher mean activities of GPx in calves born and raised in summer, compared to calves born in fall suggesting the condition of increased oxidative stress in the calves born and raised in summer compared to calves born in fall.

Advancement of pregnancy from day - 45 to day 0 significantly increased the plasma GPx activity in control and supplemented group of pregnant Murrah buffaloes. Further, enzyme activity was significantly lower in pregnant buffaloes that are supplemented with vitamin C compared to control group animals (Ganaie *et al.*, 2013a). The enzyme GPx is a component of the antioxidant system as it regulates hydrogen peroxide concentrations in the cell (Brummer *et al*., 2013).

2.4 Serum biochemical profile

The physiological responses of the animals to environmental stress during winter and summer, and their energy balance, showed that the seasonal heat and cold stress have profound effect on serum biochemical parameters (Nazafi *et al*., 2003; Rasooli *et al*., 2004).

Environmental stress has measurable effects on the endocrine profile of animals and influences the milk production due to altered metabolism (Aggarwal and Singh, 2010). High ambient temperature adversely affects the structure and physiology of the cells causing impaired transcription, RNA processing, translation, oxidative metabolism, membrane structure and function (Sunil Kumar *et al*., 2011).

Metabolic regulators are important in elucidating the picture of modulation in physiological mechanisms during stressed conditions and are best assessed by determining the enzymes governing various metabolic reactions in blood and serum (Kataria and Kataria, 2012). Summer stress induced oxidative stress may be reflected as disturbed physiology and altered blood biochemical profile of the animal (Kumar *et al*., 2012).

Oxidative stress is believed to play an important role in the regulation of the metabolic activity of some organs and productivity in farm animals (Celi and Chauhan, 2013). Alteration in protein metabolism in buffaloes and different livestock species and lipid metabolism in lactating buffaloes and in buffalo heifers due to heat stress have been reported (Das *et al*., 2013).

Metabolic profile gives good indication of metabolic and physiological adjustments that occur during stress conditions and are best assessed by determining the enzymes governing various metabolic reactions in plasma or serum (Gupta *et al*., 2013). At cellular level the ability to survive and adapt to the thermal stress involves biochemical responses and gene expression (Sharma *et al*., 2013).

During heat stress, reduction in the feed intake causes alteration in the thermal-neutral energy balance resulting in the majority of the affected dairy cows entering into negative energy balance. This negative energy balance is associated with a variety of metabolic changes like marked alteration in both carbohydrate and lipid metabolisms (Baumgard *et al*., 2014).

Zhang *et al*. (2014) reported that the exposure of lactating cows to moderate THI (73.9 ± 1.7) or high THI (80.3 ± 1.0) during hot summer significantly alters the serum biochemical profile.

2.4.1. Serum aminotransferases

Aminotransferases are a group of enzymes that catalyze the reversible transfer of the amino group from an amino acid to an oxo acid. ALT and AST shunt their amino acid

and oxo acid substrates into several intermediate pathways. Hepatocellular damage with the subsequent disruption of the plasma membrane allows leakage of intracellular enzymes such as ALT or AST into bloodstream. Due to half-lives of approximately 17 h for AST and 47 h for ALT, the presence of these enzymes in serum is considered as an indicator of recent hepatocyte injury (Amacher, 1998).

Singh *et al*. (2002) studied the effect of chronic selenosis in buffalo calves and recorded a significant increase in the plasma activity of both AST and ALT when the buffalo calves were supplemented with 8.45 ppm of selenium.

ALT and AST are two of the most reliable markers of hepatocellular injury or necrosis. ALT primarily exists in liver, but AST exists in various tissues like heart, liver, kidney and so on (An-Qiang *et al.,* 2009). The serum activity of AST and ALT is helpful in diagnosis of animal's welfare (Gupta *et al*., 2013).

2.4.1.1 Serum aspartate aminotransferase (AST)

Aspartate aminotransferase (formerly glutamic oxaloacetic transaminase; GOT) catalyzes the transamination of L-aspartate and 2-oxoglutatarate to oxaloacetate and glutamate. Pyridoxal-5-phosphate (PP) is required as a cofactor for the activity of this enzyme (Hoffmann and Solter, 2008).

Shashidar and Prasad (1993) recorded a significant increase in the serum AST activity in adult goats when they were supplied with 0.15-0.30 mg of selenium per kg body weight. Aspartate aminotransferase: AST (EC 2.6.1.1) is found both in cytosol and

mitochondria of hepatocytes but high tissue levels are also found in heart, skeletal muscle, kidney, brain and pancreas (Amacher, 1998).

The fat-tailed Iranian sheep exposed to heat stress showed significantly higher serum aspartate activity compared to the corresponding activity in sheep exposed to winter stress (Nazafi *et al*., 2003).

Srikandakumar *et al*. (2003) reported a significant decrease in the activity of the plasma AST in both Merino (84.67 to 30.67 IU/l) and Omani (145.00 to 85.83 IU/l) sheep during heat stress and the decrease was within the normal range. Further, they concluded that the decrease in the enzyme concentration is indicative of no damage to the liver tissue but the slowdown of the liver function when the animals were subjected to heat stress.

Study on effect of heat stress on blood biochemistry in Holstein, Jersey and Australian Milking Zebu cows reported a significant increase in the plasma aspartate aminotransferase activity (IU/L) during heat stress in all the breeds of cattle (Srikandakumar and Johnson, 2004).

Rasooli *et al*. (2004) studied the influence of seasonal variation on biochemical parameters and found significantly increased serum AST activity in HF heifers exposed to high ambient temperature. Non-significant increase in serum glutamic oxaloacetic transaminase activity was observed during spring season (38.68 ± 4.83 Units/ml) in crossbred calves compared to the enzyme activity during summer (38.44 ± 4.95 Units/ml) (Bahga *et al*., 2009).

Study of Al-Saeed *et al*. (2009) showed a significant increase in the plasma AST activity (U/l) during summer (70.9 ± 17.07) as against the winter (61.9 ± 16.6) in local cattle. They concluded that the increase in the plasma AST activity in cattle suggests the cellular damage in the liver. Study of metabolic and hematological profiles in Italian Friesian lactating dairy cows during heat stress showed a significant positive effect of THI on plasma AST activity and the supplementation of selenium did not bear any effect on its activity. Increase in AST indicated slight impairment of tissue as a consequence of oxidative effect (Calamari *et al*., 2011).

The climatic conditions did not bear any significant effect on both liver and serum AST activity in Marwari goats, but the sex of the animal significantly influenced the serum AST activity in both (moderate and extreme) climatic conditions (Sharma and Kataria, 2011). Though the season did not influence the serum AST enzyme activity significantly in crossbred cows, serum AST activity was significantly increased with advancement of pregnancy during both summer and winter season (Alameen and Abdelatif, 2012).

Chandra Bhan *et al*. (2012) recorded a significantly higher AST activity during summer compared to the spring season in young and adult Sahiwal cattle. Supplementation of 0.2 ppm of selenium did not exert any significant influence on the serum AST activity in buffalo heifers (Ganie *et al*., 2012).

Ashatsham-ul Haq *et al*. (2013) reported a significant increase in the plasma levels of AST during summer season and the supplementation of the summer stressed dairy cow with ascorbic acid and amla powder significantly declined the enzyme activity.

Pandey *et al*. (2013) in their study on hematobiochemical responses of Sahiwal cows during hot dry and hot humid environment observed a significant increase in the serum AST activity from morning (62.22 IU/ ml) to evening (62.95 IU/ ml), however, the activity of the enzyme did not vary during the hot humid condition.

A significantly higher serum activity of AST was reported in zinc fed goat kids during their growth phase and higher rate of assimilation of zinc from organic composition might be attributed to higher levels of zinc in the body fluids and higher AST activity (Devi *et al.*, 2014).

2.4.1.2 Serum alanine aminotransferase (ALT)

Alanine aminotransferase (EC 2.6.1.2) (ALT), formerly known as glutamic pyruvate transaminase, catalyzes the reversible transamination of L-alanine and 2-oxoglutarate to pyruvate and L-glutamate. ALT, along with other transaminases plays a role in amino acid catabolism and inter-organ nitrogen transport. Pyridoxal 5-phosphate (PP) is the cofactor of ALT, thus forming the active holoenzyme (Kaneko *et al*., 1997).

Shashidar and Prasad (1993) recorded a significant increase in the serum ALT activity in adult goats when they were supplied with 0.15-0.30 mg of selenium per kg body weight. Over all, serum ALT (L-alanine: 2-oxoglutalate aminotransferase) is one of the most universal markers for hepatic injury across species. ALT, when present in low concentration in peripheral circulation indicates normal cell turnover or release from nonvascular sources. The largest pool of ALT is in the cytosol of hepatic parenchymal cells and measurement of ALT is used to assess the hepatic injury (Amacher, 1998).

Serum activity of alanine aminotransferase was significantly higher in sheep exposed to heat stress as compared to the sheep exposed to cold stress (Nazafi *et al*., 2003). Bahga *et al.* (2009) reported a significant decrease in the activity of serum glutamic pyruvic transaminase during summer season (10.68 ± 0.96 Units/ml) compared to the activity during the spring season (16.44 ± 2.17 Units/ml) in growing young crossbred calves.

Plasma ALT activity (U/L) showed a significant increase during the summer season (30.8 ± 9.25) compared to its activity during the winter (25.1 ± 10.43) season in White Fulani cows. Further, this increase in the enzyme activity during summer season is indicative of deranged energy metabolism (Al-Saeed *et al.*, 2009).

Sharma and Kataria (2011) observed a significant increase in the ALT activity in serum (77.68 ± 1.75 vs. 57.41 ± 1.75 IU/L) and in liver tissue (441.31 ± 30.18 vs. 362.88 ± 30.18 IU/L) during extreme climatic conditions compared to moderate climatic conditions in Marwari goats.

Alameen and Abdelatif (2012) recorded numerically higher ALT values during summer compared to winter values indicating no influence of season on ALT activity. However, serum ALT activity significantly increased with advancement of pregnancy during both summer and winter season.

Chandra Bhan *et al*. (2012) recorded a significantly higher activity of ALT activity during summer compared to the spring season in young and adult Sahiwal cattle.

Ganie *et al*. (2012) observed that the supplementation of 0.2 ppm of selenium did not exert any significant effect on serum levels of ALT in buffalo heifers.

Study on hematobiochemical responses of Sahiwal cows during hot dry and hot humid environment by Pandey *et al*. (2013) revealed a significant increase in the serum ALT activity from morning (27.33 IU/ ml) to evening (30.60 IU/ ml) indicating an increased enzyme activity in the liver when the temperature was more in the evening hours.

The ALT activities in the serum of zinc supplemented kids were significantly higher than that of control group kids from 5 months onwards. Further, the metallo-enzyme like ALT could be considered as a bio-marker of the zinc status in the body (Devi *et al.*, 2014).

2.4.1.3 Serum alkaline phosphatase (ALP) activity

ALP (EC 3.1.3.1) is a homodimeric metalloenzymes responsible for removing phosphate groups from nucleotides, proteins and alkaloids. As the name suggests, alkaline phosphatases are most effective in an alkaline environment. ALP is found in many tissues including bone, liver, intestine, kidney, placenta and germ cell (Sarkar, 2012).

ALP belongs to hydrolases that hydrolyse the monoesters of phosphoric acid. It is made up of four isoenzymes – the placental, carcinoplacental, intestinal and a combines group of liver, renal and bone isoenzymes. ALP is mainly localized in the cellular membranes of hepatocytes and increased levels of ALP are found during altered bone

metabolism (Chandra *et al*., 2013a). Phosphatases are the key enzymes in liberating and recycling the phosphate molecules that are necessary for many fundamental biological processes (Ghosh and Roy, 2014).

Alkaline phosphatase in involved in maintaining the homeostasis and energy generation in animal body (Wasserman *et al*., 1996). Activity of serum ALP did not vary significantly in Iranian sheep exposed to heat and cold stress. But, the enzyme activity in these stressed groups was significantly lower than the corresponding values in animals kept under optimum temperature (Nazafi *et al*., 2003).

Abeni *et al.* (2007) recorded a significant decrease in the plasma alkaline phosphatase activity in lactating Holstein cows during two successive summer season and further they opined that this enzyme activity could be used as a quick and reliable heat stress blood marker.

Alkaline phosphatase is involved in energy metabolism and is an indicator of alkalosis and stress. Serum alkaline phosphatase activity increased non-significantly during summer compared to spring season in crossbred calves. Higher serum alkaline phosphatase activity during summer could maintain homeostasis and generate energy in the animal body during heat stress (Bahga *et al*., 2009).

Chandra Bhan *et al*. (2012) recorded a significantly higher plasma ALP activity during summer compared to the spring season in adult Sahiwal cattle and the ALP activity showed significant positive correlation with the temperature humidity index (THI).

Ashatsham-ul Haq *et al*. (2013) studied the effect of supplementation of ascorbic acid and amla powder on hemato-biochemical parameters of crossbred dairy cows during summer season and they concluded that the plasma activity of ALP increases significantly during summer season and supplementation of cows with ascorbic acid and amla powder during summer significantly reduced these enzyme activity.

Devi *et al.* (2014) studied the effect of zinc supplementation on serum enzymatic profiles in local goat kids and observed a significant increase in the serum activity of the alkaline phosphatases in zinc supplemented group of animals suggesting that the zinc as a micro-mineral stimulates the catalytic function and renders the structural stability to the enzyme.

Plasma ALP concentration declined during the 2^{nd} and 3^{rd} day of heat stress followed by sharp increase on the 4^{th} day of the heat stress and this decline in plasma ALP might be due to an increase in the plasma volume as a result of heat shock so as to maintain the homeothermy (Abdel-Fattah, 2014).

2.4.2 Serum Proteins

The plasma total protein concentration was significantly higher in sheep exposed to cold stress than the sheep exposed to heat stress (Nazafi *et al*., 2003). Rasooli *et al*. (2004) reported a significant increase in the concentrations of serum total protein and albumin during summer compared to winter in Holstein heifers. This increment in the serum protein concentration indicated a loss of extracellular fluid due to heat exposure. They also found significant positive correlation between environmental temperature and the serum concentration of total protein and albumin.

Gudev *et al*. (2007a) reported significant decline in the plasma total proteins in lactating buffaloes when they are exposed to direct solar radiation during afternoon compared to morning values. The observed decline indicates that the animals were in the stage of hemodilution. Shrikhande *et al.* (2008) observed higher level of total serum protein in lactating cows during summer season (7.22 g/dL) compared to the rainy season values (7.05 g/dL). But, the serum albumin levels were elevated during winter (3.30 g/dL) and summer season (3.28 g/dL) and declined during rainy season (3.14 g/dL). This variation in serum albumin levels might be due to various physiological, managemental and genetic factors.

Plasma total protein concentration (g/dL) significantly declined during summer season (5.63 ± 0.27) compared to the winter season (7.50 ± 0.43) in local breed of cattle. Further, the plasma concentration of albumin (g/dL) and globulin (g/dL) was also significantly reduced during summer season (2.63 ± 0.20 and 3.0 ± 0.7) as compared to the winter season (4.25 ± 0.12 and 3.25 ± 0.31) values (Al-Saeed *et al.,* 2009).

Supplementation of the buffaloes with only zinc and zinc plus vitamin E and selenium did not significantly influence the serum concentration of albumin but, significantly enhanced the serum total protein concentration compared to their control counterparts (Hala *et al*., 2009). Omran *et al*. (2011) reported a significant reduction in the plasma total protein and albumin concentration during heat stress in Egyptian buffalo calves.

Najdi rams exposed to heat load of hot summer showed significant increase in the serum concentrations of total protein (g/dL) and globulin (g/dL) compared to the rams

exposed to winter season (5.63 ± 0.26 vs. 4.91 ± 0.21 and 2.22 ± 0.28 vs. 1.53 ± 0.21). However, significant reduction in the serum concentrations of albumin in heat stressed rams was observed compared to the winter group rams. It was concluded that the increase in the serum total protein and globulin could be due to dehydration caused by enhanced breathing rate (Al-Haidary *et al*., 2012).

Ganie *et al*. (2012) observed that the supplementation of 0.2 ppm of selenium significantly increased the serum globulin levels and significantly lowered serum albumin levels in supplemented groups. However, the serum total protein concentration did not vary significantly between the control group and supplemented group of buffaloes.

Study on biochemical profile in summer stressed Beetal goats reported that the mean plasma total protein concentration did not differ significantly between summer stressed goats and pre-summer values. Further, the supplementation of summer stressed goats with vitamin C and E-Se did not influence their plasma total protein concentration (Kumar *et al*., 2012).

In Nili-Ravi buffaloes, the plasma concentration of albumin, globulin and total protein did not vary significantly between the groups and also between the seasons indicating that the buffaloes were unaffected by the heat stress and treatment with niacin did not bear any effect on total protein levels (Das *et al*., 2013).

Sharma and Puri (2013) studied the effect of temperature on biochemical profile of Marwari goats and found significant decrement in serum globulin and total protein concentrations during moderate and extreme hot conditions in female Marwari goats.

Study on the impact of short term exposure on biochemical and endocrine responses in Malpura ewes reported significant reduction in the plasma total protein concentration in the animals exposed to higher temperatures as compared to the animals exposed to lower temperature and similar trend was observed for plasma albumin levels also (Sejian *et al.,* 2013).

Heat stress reduced the concentrations of plasma total protein, albumin and globulin during first three day of exposure in both Balady and Damascus breeds of goats followed by a pronounced increase in their concentration on the 4^{th} day of exposure to heat stress. This change in the plasma protein concentration could be due to fluid shift between the compartments of the organism that play an important role in homeostasis during elevated ambient temperature (Abdel-Fattah, 2014).

2.4.3 Serum lipid profiles

Heat exposure induced hyperthermia disturbs the lipid metabolism in all animals and reduction in the concentration of plasma cholesterol and phospholipids occur in heat stressed cattle (O' Kelly, 1987).

2.4.3.1 Serum triglycerides

Though the plasma triglycerides concentration did not show any significant difference between the sheep exposed to heat stress and cold stress, the concentration in these animal group was significantly lower than the animals kept at optimum temperature (Nazafi *et al*., 2003).

Avendano-Reyes *et al*. (2010) compared three cooling systems in alleviation of heat stress in Holstein cows during hot and dry ambient conditions and reported non significant differences in the serum concentrations of triglycerides among animals of different treatments. Omran *et al*. (2011) studied the responses of Egyptian buffalo calves to heat stress and reported significant decline in the plasma triglycerides concentration in heat stressed buffalo calves as compared with their control counterpart reared under thermoneutral zone. A mean triglyceride concentration of 1.27 ± 0.01 mmol/L was recorded in Marwari goats when they were exposed to moderate ambience. But, the serum triglycerides concentration was significantly ($P<0.05$) reduced when the same animals were exposed to hot and cold ambience (Pandey *et al*., 2012).

The plasma triglycerides concentration in summer stressed goats did not differ significantly from the corresponding values of pre-summer period in Beetal goats and supplementation of the stressed goats with vitamin C and E-Se also did not bear any influence on the plasma triglyceride levels (Kumar *et al*., 2012). Alameen and Abdelatif (2012) reported significantly higher concentrations of serum triglycerides during winter in all physiological states in crossbred dairy cows.

2.4.3.2 Serum total cholesterol, HDL cholesterol, LDL-cholesterol and VLDL cholesterol

Serum cholesterol level in the animal body is influenced by several genetic and non-genetic factors such as season, age, sex, etc. The season exerts a significant effect on serum cholesterol levels in both Jersey and Red Dane cattle. Significant increase in the serum concentration of cholesterol was recorded during the season of high environmental

temperature and higher cholesterol levels may be due to depressed activity of the thyroid gland (Sinha *et al*., 1981).

Rasooli *et al*. (2004) reported a significant decline in the serum cholesterol concentration in Holstein heifers during summer season as compared to the winter season and the cholesterol level also showed significant negative correlation with mean ambient temperature. Abeni *et al.* (2007) recorded a significant decrease in the plasma cholesterol concentration in lactating Holstein cows during the hot periods of two consecutive summer season.

Lactating Bulgarian buffaloes exposed to maximum heat load when THI was 77.83, showed significant decline in the plasma cholesterol compared to the corresponding values during morning. Decline in cholesterol was attributed to the decrease in the feed intake in the hot environment and consequent reduction in intake of dietary cholesterol (Gudev *et al*., 2007a). Avendano-Reyes *et al*. (2010) in their study of three cooling systems to alleviate the heat stress in Holstein cows during hot and dry ambient conditions reported that the cooling system did not show any significant influence on the serum cholesterol concentrations.

Cincovic *et al*. (2011) reported a significant difference in the serum concentration of cholesterol in Holstein cows exposed to moderate to high heat stress compared to the cholesterol levels in those animals that were reared under thermoneutral environment. Study on heat stress responses in Egyptian buffalo calves reported a significant decline in the plasma cholesterol levels in heat stressed group against the animals of thermoneutral zone (Omran *et al*., 2011). Alameen and Abdelatif (2012) observed significantly higher

concentrations of serum cholesterol during winter in crossbred dairy cows during different stages of pregnancy.

Study on metabolic response of Marwari goats to ambient stress by Pandey *et al*. (2012) reported a mean serum cholesterol concentration of 3.3 ± 0.03 mmol/L during moderate ambience and the same was significantly lowered during hot ambience and significantly increased during cold ambience. Kumar *et al*. (2011) reported a significant increase in the plasma cholesterol concentration of buffaloes during summer stress when compared to pre-summer values and the plasma cholesterol concentration of control group and the group treated with vitamin C and E-Se did not differ significantly.

Sejian *et al*. (2013) in their study on impact of short term exposure on Malpura ewes reported a significant variation in the total plasma cholesterol concentration between different temperature treatments. The highest total cholesterol concentration was recorded when ewes were exposed to highest temperature (42 °C) while the lowest when exposed to lowest temperature (23 °C). Further, they opined that the highly significant increase in circulating cholesterol might be to support the hepatic gluconeogenesis to supply the glucose for the adaptive mechanisms.

During hot dry and hot humid season significantly higher plasma cholesterol and HDL cholesterol concentration were recorded in treatment group buffaloes (80.07 mg/dL and 31.63 mg/dL) as compared to their control (62.83 mg/dL and 24.20 mg/dL) counter parts. The change could be either due to the effect of nutrient supplementation or due to the combined effect of management factors (Das *et al*., 2013).

Holstein cows supplemented with chromium had significantly higher concentration of serum cholesterol than those without chromium supplementation during higher THI (80.3 ± 1.0) period but not during low THI (56.4 ± 2.5) and moderate THI (73.9 ± 1.7) period (Zhang *et al*., 2014).

2.5 Blood glucose

The study on effect of hot summer climate on milk yield and blood biochemistry in Friesian cows revealed a significant reduction in the plasma glucose concentration during summer compared to winter season (Habeeb *et al*., 1996). Srikandakumar *et al*. (2003) in their study on the effect of heat stress in two breeds of sheep observed significant increase in the plasma glucose in Merino sheep but significant reduction in the plasma glucose in Omani sheep. They attributed the same to greater mobilization of body fat reserves in Merino sheep in response to increased demand associated with higher respiratory rate and possible reduced feed intake, as compared to the less fat Omani sheep.

Effect of heat stress in Holstein, Jersey and Australian Milking Zebu cows revealed significant increase in the plasma glucose concentration in Holstein and Australian Milking Zebu cows but, significant decrease in plasma glucose concentration in Jersey cows during hotter month of the year (Srikandakumar and Johnson, 2004).

Significantly lower concentrations of serum glucose was recorded during summer than winter in Holstein heifer and further, the serum glucose levels showed a significant negative correlation with mean environmental temperature. Significantly higher serum

levels of glucose during winter was ascribed to augmentation of thyroid activity and metabolic rate accompanied with high levels of blood metabolites (Rasooli *et al*., 2004).

In a study to assess the influence of season and nutrition on oxidative status in Red Syrian goats, Trana *et al*. (2006) observed a significantly higher plasma glucose concentration during summer than during spring season. Abeni *et al.* (2007) recorded a significant decrease in the plasma glucose concentration in the lactating Holstein cows during the hot season.

The average blood glucose level was higher (47.98 mg/dL) during summer season whereas lowest level (44.20 mg/dL) was recorded during rainy season in lactating cows (Shrikhande *et al*., 2008). Significant variation in the serum glucose concentration during different season was observed in crossbred calves. Calves showed a significant increase in the serum glucose concentration during spring season (51.69 ± 4.40 mg/dL) compared to the concentration during summer season (38.62 ± 4.81 mg/dL) (Bahga *et al.*, 2009).

Al-Saeed *et al*. (2009) studied the effect of season on blood biochemical parameters in local cows of Iraq and recorded a significant reduction in the plasma glucose concentration (mg/dL) during summer season (55.09 ±10.0) compared to the concentration during winter season (70.68 ± 15.0). Higher concentration of plasma glucose during winter season could be due to enhanced feed intake during winter compared to summer season.

The heat stress markedly alters glucose homeostasis in affected cows. Glucose disposal into cells increases in heat stressed cows compared to pair-fed thermal neutral

cows (Behl *et al*., 2010). Avendano-Reyes *et al*. (2010) reported a significantly higher concentration of serum glucose (48.41 mg/dL) in control Holstein cows compared to the corresponding values in animals that were exposed to cooling systems during hot and dry ambient conditions (44.9 mg/dL).

The mean values of serum glucose in heat stressed cows were significantly different from the values obtained for the cows in the thermoneutral zone (Cincovic *et al*., 2011). The study of Calamari *et al.* (2011) on the effect of selenium supplementation in heat stressed lactating dairy cows showed a significant negative correlation between the THI and the plasma glucose concentration.

Al-Haidary *et al*. (2012) in their study on Najdi rams found a significant increase in the serum concentrations of glucose (mg/dL) in heat stressed rams compared to their counterparts exposed to winter season (95.07 ± 2.16 vs. 71.29 ± 2.80). Further, they opined that the observed increase could be due to stress induced activation of cortisol secretion and consequent stimulation of gluconeogenesis and inhibition of cellular glucose uptake and utilization.

Significant decline in the plasma glucose concentration was noticed during summer stress compared to the corresponding pre-summer values indicating the adverse effects of summer stress. The plasma concentration was significantly higher in heat stressed vitamin C supplemented groups compared to summer stressed vitamin C and E-Se supplemented groups indicating the beneficial effects of vitamin C supplementation (Kumar *et al*., 2012).

Pandey *et al*. (2012) showed significant decline and significant increase in serum glucose levels when Marwari goats were exposed to hot and cold ambience, respectively. But, a mean serum glucose concentration of 3.63 ± 0.03 mmol/L was recorded when they were exposed to moderate ambient stress.

Sreedhar *et al*. (2013) studied the effect of tropical environment on serum biochemical profile of Sahiwal and Jersey X Sahiwal cows. In their study, the serum glucose levels (mg/dL) of Sahiwal heifers, cows and Jersey × Sahiwal cows ranged from 61.90 ± 1.34 to 97.32 ± 0.63, 58.61 ± 1.20 to 96.90 ± 0.65 and 59.26 ± 0.58 to 113.33 ± 0.71, respectively, during their adaptability.

Chandra *et al*. (2013b) supplemented vitamin E at the rate of 1000 IU/day/cow along with zinc at the rate of 60 ppm/day/cow to Sahiwal cows and observed significant ($P<0.05$) increase in plasma glucose concentration during prepartum, at parturition and postpartum period compared to the control group cows.

Pandey *et al*. (2013) observed significant decline in blood glucose levels in Sahiwal cattle during evening hours compared to morning hours of hot dry season. They opined that the decrease could be due to the energy required by the animal to combat the stress during hot humid condition as there was increase in the THI values as well as the relative humidity. Ashatsham-ul Haq *et al*. (2013) observed significantly higher concentration of plasma glucose concentration in crossbred dairy cows when they were exposed to summer season and the supplementation of the diet with ascorbic acid and amla powder reduced the levels of plasma glucose significantly.

Serum glucose concentration of Holstein cows was significantly lower during moderate THI period and high THI period compared to low THI period. Further, the chromium supplementation did not affect the serum concentrations of glucose during all periods (Zhang *et al*., 2014).

2.6 Heat stress and endocrine disturbances

Endocrine system plays an important role in animal adaptation to thermal stress and thermal stress exerts a profound effect on circulating hormones (Johnson and Vanjonack, 1976). Average daily THI values were negatively correlated with plasma adrenal cortex hormones (corticoids) in shaded cows, plasma thyroid hormone in shaded and non-shaded cows (Ingraham *et al*., 1979).

Pituitary, thyroid and adrenal glands are known to play a crucial role in the thermoregulatory and metabolic functions of lactating dairy cattle when they acclimate to environmental heat stress (Johnson *et al*., 1988). Acclimation to heat stress has been shown to bring about several adjustments in the thermogenic pathway and endocrine functions. Thyroid hormones play an important role in adaptive thermogenesis and their plasma concentration decrease after acclimation to the heat (Shido and Sakurada, 1993). Thyroxine, growth hormone, triiodothyronine, and glucocorticoids concentration decrease in cattle exposed to heat stress (Itoh *et al*., 1998).

Thyroid hormones are considered as important regulators of the animal metabolism and serum triiodothyronine, thyroxine and cortisol concentrations have been shown to undergo significant changes during heat and cold stress (Nazafi *et al.*, 2003). Environmental stress has measurable effects on the endocrine profile of animals.

Metabolic hormones such as thyroxine, triiodothyronine and insulin are the indicators of altered metabolism during different season. Plasma cortisol levels have been used as a physiological marker of the heat stress and its concentration declines during heat acclimation to reduce the heat production (Aggarwal and Singh, 2010).

Exposure of animals to heat stress stimulates the hypothalamo-pituitary-adrenal axis and hence concentration of hormones such as thyroxine, cortisol, and prolactin could be used as indicators of stress in animals (Sivakumar *et al*., 2010).

Metabolic hormones such as thyroxine, triiodothyronine and insulin can be used to indicate the metabolic changes that occur during different seasons (Aggarwal and Singh, 2010). Environmental stressor like thermal stress potentially activate the hypothalamo-pituitary-adrenal cortical axis (HPA) and sympatho-adrenal medullary axis which in turn mediate the hormonal changes that occur during thermal stress (Sunil Kumar *et al*., 2011; Minton, 1994).

Physiological mechanism including endocrine responses and cellular heat stress responses were triggered to maintain homeostasis when the animals were exposed to heat stress (Sharma *et al*., 2013). Climatic factors or seasonal changes greatly influence the behavior of animals due to neuroendocrine response to climatic elements, consequently affecting production and health of animals (Sejian *et al*., 2013). There is increase in plasma concentration of cortisol and corticosterone and less frequently an increase in plasma epinephrine and nor epinephrine concentration in stressed animals (Nisar *et al*., 2013).

Reduced feed intake during heat stress brings about numerous changes in the digestive tract, acid-base chemistry, and hormone activity (West, 2003). Heat stress increases the body temperature, pulse rate, and respiratory rate that lead to marked reduction in feed intake, redistribution in the blood flow, depression of immune system and alteration in endocrine functions which ultimately affect the productivity and reproductive performance in the livestock (Al-Samawi *et al*., 2014).

2.6.1 Plasma thyroid hormones profile during heat stress

The study on seasonal effect of tropical climate on shaded and non-shaded cows by Ingraham *et al*. (1979) revealed that the THI decreased from 75 to 70 during September to December and the average THI values were negatively correlated with plasma corticoids in shaded cows, plasma thyroid hormone in shaded and non-shaded cows.

Magdub *et al*. (1982) reported that during the heat stress there was a significant reduction in the concentration of triiodothyronine and thyroxine in plasma and milk of lactating cows. Plasma thyroxine concentrations were lower in heat stressed cows (51.2 vs. 66.4 ng/mL), while the plasma triiodothyronine concentrations were elevated (1.8 vs. 1.5 ng/mL), indicating altered thyroid hormone metabolism in heat stressed cows (Collier *et al*., 1982).

Acclimation to the heat stress suppresses the oxygen consumption by decreasing the plasma thyroid hormonal levels. Thus reduction in the heat production in heat-acclimated rats could have an intimate association with the plasma thyroid hormone levels (Shido and Sakurada, 1993). The study of Habeeb *et al*. (1996) revealed a

significant reduction in the plasma thyroxine and significant increase in plasma triiodothyronine levels in Friesian cows during summer season compared to the winter season.

A study on circadian variations in the plasma levels of thyroid hormones in cattle and buffaloes during summer and winter recorded a plasma T_4 peak of 39.42 ± 3.61 ng/ml during day time in the winter and a plasma T_4 peak of 33.89 ± 2.39 ng / ml during day time in summer season in buffaloes. In cattle the peak plasma levels of thyroxine were 39.27 ± 9.70 ng / mL during day time in winter and 30.90 ± 5.26 ng / ml during day time in summer (Aggarwal *et al*., 2003).

Nazafi *et al*. (2003) observed significantly higher concentration of serum triiodothyronine and thyroxine during cold stress compared to their heat stress values in Iranian flat-tailed sheep. A study of seasonal influence on thyroid activity and biochemical profiles in Holstein heifer reported a significant reduction in the serum concentrations of both T_3 and T_4 during summer as compared to winter. But, only T_3 showed significant negative correlation with mean environmental temperature (Rasooli *et al.*, 2004).

Physiological response to heat stress is a reduction in the heat production which in turn, is caused, in large by a reduction in the feed intake, milk yield and thyroid hormone secretion (Hansen, 2004). Mean plasma concentration of T_4 varies between 15 and 44 ng/mL and that of T_3 varies between 0.64 ng / mL and 1.0 ng/ml in animal species of veterinary importance. Mean concentration of T_4 and T_3 has been reported as 62 ng / mL and 0.92 ng / ml, respectively in bovines (Eiler, 2005).

The major exogenous regulator of thyroid gland activity is the environmental temperature and an inverse relationship between ambient temperature and blood thyroid hormone concentrations has been found in sheep (Todini, 2007). Hala *et al*. (2009) observed a significant increase in the serum concentrations of T_3 in buffaloes fed with zinc methionine and in animals fed with vitamin E/Se + Zn methionine compared to their control counterparts. They also reported a numerical depression in the serum concentration of T_4 in treatment group compared to the control group buffaloes. They concluded that the selenium being the integral part of the selenocysteine enzyme 5-triiodinase is needed for the hepatic conversion of T_4 to T_3.

Aggarwal and Singh (2010) reported a significantly higher concentrations of plasma thyroxine in wallowing buffaloes compared to the buffaloes under shower during both hot humid (50.57 ± 0.61 vs. 48.25 ± 0.54 ng/ml) and hot dry (52.57 ± 0.67 vs. 50.65 ± 0.50 ng/ml) season. During heat stress, different cooling management systems in lactating Holstein cows showed no significant influence on the serum concentration of the T_3 and T_4 (Avendano-Reyes *et al*., 2010).

Significant reduction in the plasma concentration of T_3 (4.55 ± 0.16 vs. 3.21 ± 0.08 pmol/L) and T_4 (21.27 ± 0.51 vs. 16.70 ± 0.19 Pmol/L) was recorded in heat stressed goats compared to their control counterparts and dietary supplementation alleviated the negative effect of heat stress on thyroid hormones (Sivakumar *et al*., 2010).

Study on heat stress response in buffalo calves revealed significant reduction in plasma thyroxine (µl/dL) and triiodothyronine (ng/dL) concentrations in heat stressed group animals as compared to animals under thermoneutral zone (Omran *et al*., 2011).

Alameen and Abdelatif *et al*. (2012) reported that the serum concentrations of triiodothyronine was significantly higher and that of thyroxine was significantly lower during summer season when compared to their values during the winter season in crossbred dairy cows.

Plasma T_3 and T_4 concentration showed significant variation among the different breeds of goats with higher mean values observed in cold adapted breeds (Gaddi and Chegu) than heat adapted goat breeds (Sirohi and Barbari). The levels of both the thyroid hormones were higher during winter and lower during summer in all the goat breeds. The higher concentrations of thyroid hormones in cold adapted goat breeds than heat adapted breeds indicated breed variation in adaptation to different climatic conditions (Banerjee *et al*., 2013a).

Sejian *et al.* (2013) studied the impact of short term exposure to various temperatures on endocrine responses in Malpura ewes and recorded a significant increase in the plasma levels of T_3 and T_4 in the animals exposed to a temperature of 23 °C, decrease in the concentration of T_3 in the animals exposed to 40 °C and in the animals exposed to 42 °C when compared to control animals.

Al-Samawi *et al*. (2014) studied the effect of environmental heat stress in female Aardi goats and recorded significant reduction in serum concentrations of T_3 (1.37 ± 0.03 ng/ml and 1.61 ± 0.07 ng/mL) and T_4 (4.81 ± 0.13 μg/mL and 5.56 ± 0.13 μg/mL) during summer compared to winter, respectively. Further, they opined that the reduction in thyroid hormone levels is to reduce the metabolism so as to decrease heat production and heat load in the animal body.

Das *et al*. (2014) reported a significant decrease (17.2%) in the plasma T_3 concentration in lactating buffaloes with increase in the ambient temperature from 17.5 to 37.1 °C. However, they observed non-significantly higher concentration of T_4 in control group buffaloes as compared to treatment group (niacin and yeast) animals during hot dry and hot humid season. Study indicated that the diet supplementation with antioxidants has beneficial effect during hot dry and hot humid summer without affecting the T_3 and T_4 profile.

2.6.2 Plasma cortisol concentration during heat stress

Cortisol is a major glucocorticoid secreted from the adrenal cortex under the influence of an adenohypophyseal hormone adrenocorticotropic hormone (ACTH) which in turn is regulated by hypothalamic hormone corticotrophin releasing hormone (CRH). Various stressors (thermal, transportation, weaning etc.) are reported to activate the hypothalamo-pituitary-adrenal (HPA) axis in domestic farm animals resulting in increased synthesis and release of cortisol (Minton, 1994).

The increase in the plasma cortisol levels, as a consequence of the activation of the hypothalamic-pituitary-adrenal axis, is one of the best known and consistent neuroendocrine responses to stress (Sevi *et al*., 2002).

The serum cortisol concentration did not show any significant difference in heat and cold stressed Iranian sheep (Nazafi *et al*., 2003). The study on lactating buffaloes exposed to heat stress by Gudev *et al*. (2007b) indicated that the heat stress was not associated with the enhancement of plasma cortisol levels and the same could be due to

hormonal integration and modulating effect of hypothalamic-pituitary-adrenal axis on other endocrine glands involved in the maintenance of thermal homeostasis.

At temperature above or below thermo neutral zone, corticosteroid secretion increases in response to stress. By decreasing the synthesis and secretion of corticosteroids, vitamin C alleviates the negative side effects of stress (Ramanath *et al*., 2008).

Bhat *et al*. (2008) reported significantly higher concentrations of plasma cortisol in Wistar albino rats exposed to high ambient temperature and humidity of the hot season (4473.96 ± 1060.03 nmoles/L) as compared to the rats that were exposed to cold season (3663.53 ± 986.21 nmoles/L).

Significant increase in the serum concentration of cortisol was observed in buffalo cows exposed to summer stress compared to winter group animals and the same was significantly declined when the summer group animals were treated with antioxidants (Megahed *et al*., 2008). The plasma cortisol concentration was significantly lower in group II buffaloes (allowed to wallow) compared to group I buffaloes (kept under shower) during both hot humid (2.64 vs. 4.33 ng/ml) and hot dry (4.80 vs. 2.60 ng/ml) season (Aggarwal and Singh, 2010).

Sunil Kumar *et al. (*2010) reported that the plasma cortisol significantly increased during hot dry conditions in both control and treatment group (sodium bicarbonate, potassium carbonate and ascorbic acid polyphosphate) of buffaloes. But the increase was significantly lesser in treatment group animals compared to the control animals.

Sivakumar *et al.* (2010) reported a significant increase in the plasma concentration of cortisol (nmol/L) in Black Bengal goats subjected to heat stress compared to control animals (40.57 ± 0.92 vs. 25.27 ± 0.65). They also observed significant reduction in the plasma cortisol concentration in treatment group during heat stress, indicating the amelioration of heat stress due to supplementation of vitamin C, vitamin E and selenium.

Gradual increase in the plasma cortisol concentration was observed up to 240 days of pregnancy and it showed a sharp decline on 280 and 290 days of pregnancy in group I (negative control), group II (Single injection of vitamin E+Se) and group III (Four injections of vitamin E + Se) buffaloes. However, the plasma cortisol values on 280 and 290 days of pregnancy were significantly lower in treatment groups (group II and III) compared to the control animals (group I) (Dimri *et al*., 2010).

Soltan (2010) reported a significant increase in the serum cortisol levels in the lactating dairy cows when exposed to heat stress and the study also observed a significant reduction in the serum cortisol concentrations in animals fed with chromium @ 6 mg/head/day. Hypothalamo-pituitary-adrenocortical (HPA) axis is activated in response to stressors like heat and inflammation resulting in increased blood cortisol levels (Mete *et al*., 2012). Chandra Bhan *et al*. (2012) reported significantly higher plasma cortisol levels in growing and adult Sahiwal cattle during summer (8.91 ng / ml) over the spring (1.92 ng / ml) season and further they observed a significant positive correlation between the plasma cortisol concentration and the THI.

Sharma *et al.* (2013) in their study of influence of melatonin administration on thyroid and cortisol hormone levels in goats observed significant increase in concentration of cortisol with increase in the exposure to temperature in the control group. Further, at all the exposure temperatures, the cortisol levels in treatment group were significantly lower compared to the control group. Significant rise in the serum cortisol levels in control group in the study indicated the effect of thermal stress on goats and reduced cortisol concentration in treated group of animals indicated the stress relieving effect of melatonin.

Banerjee *et al.* (2013a), in their study, reported a significant variation in the plasma cortisol levels between heat adapted and cold adapted breeds of goats and the plasma cortisol levels were higher in heat adapted goats than the cold adapted breeds. The difference in the cortisol levels of different breeds was ascribed to the adaptation of the heat and cold adapted goats to different climatic conditions. The varying levels of cortisol help in physiological adjustment to the environment and enable the animal to tolerate the stressful condition.

Study of Sejian *et al.* (2013) on short term exposure of Malpura ewes to various temperature showed a significant decrease in the plasma levels of cortisol in the animals exposed to a temperature of 23 °C and increase in the concentration of plasma cortisol in the animals exposed to 40 °C and in the animals exposed to 42 °C when compared to control animals.

Khongdee *et al*. (2013) studied the effect of modified roofing in alleviating heat stress in young male swamp buffaloes during hot humid conditions and observed a

significant reduction in the plasma cortisol levels (2.14 ± 0.24 ng/ml) in buffaloes kept under modified roof as compared to their values (3.38 ± 0.37 ng/ml) in buffaloes reared under normal roofing. Study concluded that the roof modification facilitated the reduction in the heat load and was an effective mean of alleviating the heat stress during hot humid conditions.

Plasma cortisol, the physiological markers of stress, is reduced during heat acclimatization and helps the animal in reducing heat production. It elicits physiological adjustments, which enable the animals to tolerate the stressful condition (Das *et al*., 2014).

Study of environmental heat stress on Aardi goats revealed a significant increase in the serum cortisol concentration during summer season (26.29 ± 1.04 ng/ml) than during winter season (20.27 ± 1.15 ng/ml) and such an increase during summer could be due to activation of hypothalamo-pituitary-adrenal axis due to heat stress during hotter months (Al-Samawi *et al*., 2014).

2.6.3 Heat stress and plasma insulin levels

Plasma insulin showed declining trend during early and mid-stages of lactation when exposed to heat stress, whereas, at the late-stage of lactation insulin concentrations were highest and showed no indication of decline due to temperature. In fact, post-heat stress concentrations of insulin at all stages of lactation were higher than the corresponding values during heat stress. This might have been due to a temporarily lowered voluntary intake of feed during heat (Johnson *et al*., 1988).

A study on insulin and glucagon secretion in lactating cows during heat exposure indicated significant increase in basal insulin concentrations in heat stressed animals compared to animals of thermoneutral zone (Itoh *et al*., 1998).

Daily plasma basal insulin showed interaction between the group and period, they did not differ in cows under thermoneutral conditions fed *ad libitum* but significantly increased in heat stressed cows fed *ad libitum* and in heat stressed and recombinant bovine somatotropin administered cows (Wheelock *et al*., 2010).

Study of Omran *et al*. (2011) on heat stress responses in Egyptian buffalo calves showed significant decline in the concentration of plasma insulin in animals exposed to the ambient temperature of 40 °C as compared to the animals reared under comfortable zone. Aggarwal and Singh (2010) reported a significant difference in the plasma levels of insulin between two groups during two different seasons. During hot-dry season and hot-humid season the plasma insulin levels (μU/mL) were significantly higher in buffaloes (10.86 ± 0.27 and 9.62 ± 0.30) that were allowed to wallow compared to the animals that were kept under shower (8.30 ± 0.27 and 7.86 ± 0.33).

2.7 Heat stress and immunity

Heat stress reduces the responsiveness of the peripheral blood mononuclear cells to mitogen and thus impairs acquired immune function as measured by lymphocyte proliferation (Lacetera *et al*., 2006). Thermal exposure of buffaloes was associated with declined lymphocyte proliferative response and IL-2 production, indicating the reduced immune status of buffalo heifers (Patir and Upadhyay, 2007).

Immune cells are particularly sensitive to oxidative stress because their membranes contain high concentrations of polyunsaturated fatty acids that are very susceptible to peroxidation and their production of large amounts of ROS when stimulated (Spears and Weiss, 2008). High ambient temperature or summer heat stress is a major contributing factor to low fertility and immunity among the farm animals (Hala *et al*., 2009). Oxidative stress may be the major underlying cause of inflammatory and immune dysfunction in dairy cattle (Sordillo and Aitken, 2009).

The mechanism whereby heat stress affects immune status may be mediated through changes in prolactin signaling pathways. The greater concentrations of prolactin in plasma of cows exposed to heat stress were associated with reduced lymphocyte proliferation compared with cows maintained in cool climate (Amaral *et al.*, 2010).

Heat stress and photoperiod both affect milk production and immune status in dairy cows (Amaral *et al*., 2011). Heat stress in dry cows results in reduced immune function and the affected animals are at higher risk for disease compared with cows that are cooled when dry (Dahl, 2012). Heat stress suppresses different components of the immune system and enhances the animal susceptibility to various diseases. Some of the responses of heat stress can be prevented by alternative management practices and nutritional strategies (Aggarwal and Upadhyay, 2013). Serum insulin concentrations before lipopolysaccharides (LPS) administration were not affected by the ambient temperature, but significant differences in the concentrations were noticed between the breeds. Specifically, insulin concentrations before LPS challenge were greater in heat-

tolerant Romosinuano heifers than in Angus *Bos taurus* breed heifers (Burdick Sanchez *et al*., 2013).

2.7.1 Serum immunoglobulin levels during heat stress

The values of total immunoglobulin concentration significantly varied between the medium and high body condition score animals. In medium body condition score cows total immunoglobulin levels were 27.94 ± 0.85 and 24.70 ± 0.82 mg/ml at 200 days prior to calving and on the day of calving. But, in high body condition score animals mean ± SE values of total immunoglobulin were 34.76 ± 1.57 and 27.90 ± 1.25 mg/ml at 200 days prior to calving and on the day of calving (Aggarwal *et al*., 2008).

Study of Hala *et al*. (2009) elucidated that the buffaloes supplemented with zinc in combination with vitamin E / Se had significantly higher levels of serum immunoglobulins (gamma-globulins) than those that received zinc only and those that did not receive any supplementation. Further, they concluded that this change in the immune status in supplemented group can be ascribed to synergistic effect of vitamin E and selenium on immunity.

The plasma total immunoglobulin levels in both control and treatment crossbred cows decreased steadily up to day 5 (postpartum), and after that, values increased from day 10 to day 20 (postpartum). However, in treatment group that received alpha-tocopherol acetate, the immunoglobulin concentration on all the observation days was significantly higher than corresponding values in their counterpart (Aggarwal *et al*., 2013).

Materials and Methods

III. MATERIALS AND METHODS

3.1. Location of the study area

The present study was conducted at Madabal Village of Magadi Taluk, Ramanagara District, was located between the latitude 12° 58' 0" and 12° 97’ 0" degrees north and longitude 77° 14' 0" degrees east, at an altitude of 900 meters above the sea level.

The maximum temperature recorded over the years (past 20 years) during summer was around 38 °C and the minimum was around 12 °C in winter. The average maximum and minimum temperatures were around 33 °C and 14 °C, respectively. The average annual recorded rainfall was 800 mm and most of it was received between June and September from the southwest monsoon.

3.2 Duration of the study

Based on the rainfall, temperature and humidity, the Indian Meteorological Department, Government of India has classified the seasons as:

Winter Season : November to February

Summer Season : March to June

Rainy Season : July to October

The study was conducted in all the three seasons with two months representing each season. Study period included winter months (January and February), summer months (April and May) and rainy months (July and August). Daily minimum and maximum temperature (Degree Celsius) and daily minimum and maximum relative

humidity (percentage) were obtained for the study period (January to August 2014) from internet sources (http/www.accuweather.com).

3.3 Assessment of the heat stress

Utilizing the recorded monthly meteorological data on temperature and relative humidity, the temperature humidity index (THI) for the different months during the entire study period was calculated using the formula as mentioned by Dikmen and Hansen (2009).

$$THI = (1.8 \times T_{db} + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T_{db} - 26.8)]$$

Where: T_{db}: Average dry bulb temperature

RH: Average Relative Humidity

3.4 Animal selection

A group of twelve recently calved female Hallikar cattle that were apparently healthy, free from physical or anatomical abnormalities aged between 4 and 6 years were randomly selected from different farmers of the Madabal village of Magadi Taluk, Ramanagara District, Karnataka, India, for the present study. The selected animals were dewormed for both ectoparasites and endoparasites before the study was initiated. All the study animals were maintained in semi-intensive housing system with uniform feeding and managemental practices in the farmers' premises. Each of the study animal was fed with 8-10 kg/day of dry fodder and 4-5 kg/day green fodder and are also provided with *ad libitum* water during entire period of the study. All the animals were allowed for grazing for about 7 hours (10.00 AM to 5.00 PM) daily during the day hours. Though some of the selected animals exhibited the estrous during the study period, they were not

inseminated until the completion of the study so as to maintain the uniformity among the study animals.

Animals were divided into two groups, *viz.*, control group and supplemented group, with six animals in each group. Animals of both the groups were exposed to environmental stressors for three different periods of the year such as winter months (January and February, 2014), summer months (April and May, 2014) and comfortable months (July and August, 2014) by allowing them for free grazing during the sunny hours of the day. Animals of the control group received no supplementation, while the supplemented group received vitamin E and selenium for the entire period of the study in addition to the regular maintenance diet.

3.5 Vitamin E and selenium supplementation

Vitamin E (D-alpha tocopherol acetate) and selenium (sodium selenite) in the form of powder with 50% and 45% purity, respectively, were procured from Provimi Animal Nutrition India Pvt. Ltd., Bengaluru, India. Oral dosages of the Vitamin E and selenium were fixed as detailed below.

1. Vitamin E (D-alpha tocopherol acetate): 1000 IU/day/animal (Chandra *et al*., 2013)
2. Selenium (sodium selenite): 0.3 ppm / kg dry matter intake (NRC, 2001)

Daily requirement of both the supplements were calculated assuming that the total dry matter intake of each animal would be about 10 kg. The required amounts of vitamin

E (1.47 g/animal/day) and selenium (0.007 g/animal/day) every day was fed to the animals selected under the supplementation group.

3.6 Blood collection

Blood samples from each of the control and study group were collected during morning hours (8.00 to 9.00 A.M.) in all the three study periods. Blood samples were collected on the last day of every month in the respective period. Each time two blood samples (5 ml each) were collected from each animal, one sample in heparinized vacutainer, for plasma separation, and the other in the vacutainer coated with clot activator for serum separation. Portion of the blood collected in heparinized vacutainer was utilized immediately for whole blood glucose determination. Remaining part of the heparinized blood samples along with the coagulated blood samples were transported to the laboratory in refrigerated temperature within two hours after the collection for further processing.

3.7 Whole blood glucose estimation

Blood glucose levels were estimated immediately after the blood collection by using Gluco Chek glucometer manufactured by Major Biosystem Corp., New Taipei City, Taiwan.

3.8 Separation of blood plasma

Blood plasma was separated from heparinized blood by subjecting it to centrifugation at the rate of 700 × g for 15 minutes (Kumar *et al*., 2011). The plasma samples obtained were stored at – 80 °C in different aliquots until they were used for the

analysis of heat shock protein 70 (HSP70) and hormonal profiles (cortisol, triiodothyronine, thyroxine and insulin). The erythrocyte pack obtained after the separation of plasma was utilized immediately to prepare the hemolysate (10 %) for assessing the activity of antioxidant enzymes, *viz*., catalase, super oxide dismutase and glutathione peroxidase.

3.9 Preparation of erythrocyte-lysate for antioxidant assay

Packed erythrocytes obtained during the separation of blood plasma were utilized for the preparation of erythrocyte-lysate (hemolysate). Packed red blood cells were mixed with equal volume of chilled phosphate buffer saline (pH 7.4) and shaken gently for proper mixing and centrifuged at 700 × g for 15 minutes. Then the supernatant was removed and the cellular pack was again added with chilled phosphate buffer saline (pH 7.4) and centrifuged. This procedure was repeated three times. Finally about 100 µl of washed red cell volume was mixed with 900 µl chilled distilled water to obtain 10 % hemolysate. The obtained erythrocyte-lysates were centrifuged at 10,000 x g for 5 minutes to obtain membrane free hemolysate which was later stored at – 80 °C in different aliquots until they were analyzed for the activities of various antioxidant enzymes, *viz*., erythrocyte catalase (CAT), erythrocyte super oxide dismutase (SOD) and erythrocyte glutathione peroxidase (GPx).

3.10 Determination of hemoglobin in the hemolysate

Part of the hemolysate (10%) prepared for the determination of antioxidant enzyme activities was immediately utilized for the estimation of hemoglobin using

cyanomethemoglobin method (Bhat *et al*., 2008 and Sharma *et al.,* 2011) detailed hereunder.

3.10.1. Cyanomethemoglobin method of hemoglobin determination

Hemoglobin in the hemolysate was determined using the hemoglobin reagent (HEMOCHOR–D) kits manufactured by Crest Biosystems, Coral Clinical Systems, Goa, India, with the help of photoelectric colorimeter (Photic-20) manufactured by Labotech Equipments and Engineering Systems, Telangana, India.

Calculations: Hemoglobin in g /dl = Absorbance of the Test X 36.8

Note: The determined values of hemoglobin which were required for the calculation and expression of antioxidant enzyme activities were in the range of 1.10 – 1.88 g per cent in winter, 1.03-1.93 g per cent in summer and 1.12 – 1.78 g per cent in rainy season in control group. In supplemented group, the values were in the range of 1.26 - 1.45 g per cent, 1.56 - 1.75 g per cent and 1.23 -1.89 g per cent, respectively in winter, summer and rainy seasons.

3.11 Quantitative determination of bovine heat shock protein70 (HSP70) concentrations in blood plasma

The concentration of heat shock protein (HSP70) in the blood plasma sample was determined by ELISA method with the use of Bovine HSP70 ELISA reagent kit manufactured by CUSABIO, China, as per the protocol provided by the manufacturer.

Assay principle

Current assay is based on the competitive inhibition enzyme immunoassay technique. The competitive inhibition reaction was accomplished between the HSP70 (standards or samples) and the biotin-conjugated HSP70 by the addition of both of them to the microtiter plate (provided with the kit) that had been pre-coated with an antibody specific to HSP70. The more the amount of HSP70 in the samples, the less Biotin-conjugated HSP70 binds to pre-coated antibodies. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Substrate solution was added to the wells and the intensity of the color developed was inversely proportional to the concentration of HSP70 in the sample. The color development was stopped and the optical density of each well was read at 450 nm (blue or blue-violet).

3.12 Determination of antioxidant enzyme activities

3.12.1 Determination of catalase activity in erythrocyte-lysate

Erythrocyte catalase activity in 10% hemolysate was determined by using H_2O_2 as a substrate as per the method described by Caliborne (1985). One unit of activity is equal to the mmol of H_2O_2 degraded per minute per mg of hemoglobin.

Principle

Catalase (EC 1.11.1.6) activity was determined by monitoring the decrease in absorbance spectrophotometrically at 240 nm due to decomposition of hydrogen peroxide (H_2O_2). The difference in extinction coefficient per unit time was measured as catalase activity.

Calculation

Catalase (μmoles of H_2O_2 decomposed / g hemoglobin) = [(1/X value x ΔOD) /hemoglobin] x 5250

Where X corresponds to the number of intervals for which the OD value was taken.

Unit of activity: Enzyme activity was expressed as μmoles of H_2O_2 decomposed per minute per gram of hemoglobin.

3.12.2 Determination of superoxide dismutase activity in erythrocyte-lysate

The activity of superoxide dismutase (SOD) in the 10 % erythrocyte was determined using the method described by Marklund and Marklund (1974).

Principle

Superoxide is an intermediate anion in the auto-oxidation of pyrogallol which occurs at pH 8.2. The ability of SOD to inhibit the auto-oxidation of pyrogallol at pH of 8.2 provides the basis for enzyme activity.

Calculation

SOD (units / g Hb) = [(1/X value x ΔOD x Dilution factor) / Hemoglobin] x 625

Where, X corresponds to the number of intervals for which the OD (420 nm) value was taken.

Unit of activity: SOD activity was expressed in terms of units per minute per gram of hemoglobin. One unit of SOD was defined as the amount of enzyme required to inhibit pyrogallol auto-oxidation reaction by 50 per cent.

3.12.3 Determination of glutathione peroxidase (GPx) activity in erythrocyte-lysate

Glutathione peroxidase was determined by the method described by Rotruck *et al.* (1973).

Principle

GPx reacts with H_2O_2 and reduced glutathione giving rise to oxidoreductase which forms a colored complex with dithio bis-nitrobenzoic acid (DTNB). The intensity of color development is directly proportional to the amount of GPx present in the tissue. The intensity of the developed color was read at 412 nm.

Calculation

GPx (μmoles of GSH utilized /min /g of Hemoglobin) = [(OD Value X Dilution factor)/ Hemoglobin] x 319.35.

Unit of activity: Enzyme activity was expressed as μmoles of glutathione oxidised/min/g of hemoglobin.

3.13 Separation of serum

Blood samples collected in clot activator coated vacutainer were allowed to clot by keeping it undisturbed for 30 minutes at room temperature. Clotted blood samples were transported in refrigerated temperature to the laboratory and centrifuged at the rate of 700 × g for 15 minutes to obtain the serum. Small fraction of the serum obtained from each sample was immediately utilized for the estimation of activity of enzymes like alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine

aminotransferase (ALT). Remaining portion of the serum samples was stored at – 80 °C for further analysis of other biochemical constituents.

3.13.1 Determination of serum transaminases and alkaline phosphatase

The transaminase and alkaline phosphatase activities were determined at 37 °C with the help of Microlab 300 semi- automated biochemical analyzer supplied by Merck Pvt. Ltd, Mumbai, using commercially available reagent kits manufactured by Transasia Bio-medicals Ltd., Himachal Pradesh, in technical collaboration with ERBA diagnostics.

1. Serum aspartate aminotransferase (AST) activity was determined by ERBA Kit developed as per Tietz (1986).
2. Serum alanine aminotransferase (ALT) activity was determined by ERBA Kit developed as per Bradley *et al*. (1972).
3. Serum alkaline phosphatase (ALP) activity was determined by ERBA Kit developed as per Burtis and Ashwood (1999).

3.13.2 Determination of serum total protein, albumin and globulin concentration

The serum was utilized for determination of the serum total protein and albumin concentration using Microlab 300 semi-automated biochemical analyzer supplied by Merck Pvt. Ltd, Mumbai, with the help of commercially available reagent kits manufactured by Transasia Bio-medicals Ltd., in technical collaboration with ERBA diagnostics.

1. Serum total protein was estimated by using reagent kits developed as per the procedure described by Tietz (1986).

2. Serum albumin was estimated by using the reagent kits developed as per the procedure described by Doumas *et al.* (1971).

3. The serum globulin concentration was determined indirectly as a difference of the total protein and the albumin concentration.

3.13.3 Analysis of serum triglycerides and total cholesterol

Serum triglycerides and total cholesterol concentrations were determined using Microlab 300 semi-automated biochemical analyzer manufactured by Merck Pvt. Ltd, Mumbai, with the help of commercially available reagent kits manufactured by Transasia Bio-medicals Ltd., in collaboration with ERBA diagnostics for the respective parameters.

1. Serum triglyceride levels were estimated by using reagent kit as per the method of Allain *et al*. (1974).

2. Serum total cholesterol levels were analyzed using reagent kits based on the method of Wako and modified by McGowan (1983).

3.13.4 Serum HDL cholesterol (HDL-C)

HDL cholesterol in the serum was estimated by HDL direct kit manufactured by Spinreact, S. A. and marketed by Euro Diagnostic Systems Pvt. Ltd., Chennai, using biochemical analyzer (Merck Pvt. Ltd, Mumbai). High density lipoprotein cholesterol (HDL-C) in serum was expressed as mg/dL of serum.

3.13.5 Serum LDL cholesterol (LDL-C)

Low density lipoprotein cholesterol (LDL-C) in serum was calculated as the difference between total cholesterol and the sum of VLDL-C and HDL-C as suggested by Kanchana and Jeyanthi (2010). The results were expressed as mg/dL of serum.

LDL-Cholesterol = Total Cholesterol - (VLDL-C + HDL-C)

3.13.6 Serum very low density lipoprotein cholesterol (VLDL-C)

Very low density lipoprotein cholesterol (VLDL-C) in serum was calculated by employing the Friedwald formula and results were expressed as mg/dL of serum (Satyanarayana and Chakrapani, 2006).

$$\text{VLDL-C} = \frac{\text{Triglycerides}}{5}$$

3.14 Immunoradiometric assay of blood plasma hormones

The concentration of the hormones like triiodothyronine, thyroxine, insulin and cortisol in the blood plasma were determined by using standard radioimmunoassay procedures utilizing the respective RIA kits.

3.14.1 Radioimmunoassay of triiodothyronine (T_3), thyroxine (T_4) and insulin in the blood plasma

Triiodothyronine (T_3), thyroxine (T_4) and insulin levels in the blood plasma were estimated using the RIAK-4/4A, RIAK-5/5A and RIAK-1 radioimmunoassay kits supplied by Board of Radiation and Isotope Technology, Bhabha Atomic Research Centre, Mumbai. Standard radioimmunoassay protocol provided in the leaflet along with

each kit was followed to determine the hormone levels in the blood plasma. The radioactivity was determined by placing the tubes in the gamma counter for 60 seconds, supported with data reduction and analysis software "SAPRICAL" for PC based RIA counter model PRIA-1 (Para Electronics, Mumbai). The intra and inter assay variations were 8 and 12 per cent, respectively.

3.14.2 Immunoradiometric assay of blood plasma cortisol

Blood plasma cortisol was estimated using the RIA kit (IM1841) manufactured by Beckman Coulter, Germany. The standard RIA protocol provided along with the RIA kit was utilized to determine the plasma cortisol concentrations.

The radioactivity in the plasma sample in terms of counts per minute (cpm) was determined using the Scintillation Gamma Counter PRIA-I (Para Electronics, Mumbai), set for ^{125}I for 60 seconds. Using the cpm values, the results were obtained from the interpolation of the standard curve.

3.15 Determination of serum immunoglobulin by Polyethylene Glycol Immunoglobulin Precipitation (PIP)

The total protein levels in the serum samples were determined using Microlab 300 semi-automated biochemical analyzer (Merck Pvt. Ltd, Mumbai), using kits from ERBA diagnostics (Transasia Biomedicals Ltd. HP). The serum samples were subjected to precipitation of immune complexes by using polyethylene glycol 8000 as per the procedure described by Hartree (1972) and Slebodzinska and Slebodzinski (1982).

3.16 Statistical analysis

All the data of the present study were analyzed using computerized statistical software programme, GraphPad Prism version 5.01 (2007) by applying two-way ANOVA with Bonferroni post mean comparison test. The significance of analysis was determined at probability levels of 95 per cent ($P<0.05$).

Results

IV. RESULTS

The meteorological data recorded during the present study along with Temperature Humidity Index (THI), plasma HSP70, erythrocyte antioxidant enzyme activities, certain serum biochemical profile and plasma hormonal profile obtained during the different months of the study period of the year 2014 are presented herein.

4.1 Monthly average temperature (°C) and relative humidity (%)

The monthly minimum and maximum temperature and monthly minimum and maximum relative humidity percentage obtained from the internet source for study location is presented in Table 1.

The mean ± SE values of monthly average temperature and relative humidity during different months of the study period are presented in Table 2.

In the present study, maximum ambient temperature was recorded during the month of April (29.77 ± 0.25) and lowest temperature was recorded during January (22.34 ± 0.16). The relative humidity was maximum (74.40 ± 1.10) and minimum (42.77 ± 1.28) during the months of August and February, respectively.

4.1.1 Temperature humidity index (THI)

The values of temperature humidity index (THI) during different months of the study period are depicted in the Table 3.

During the study period, lowest THI was recorded during the winter month of January and highest THI was recorded during the summer month of April with the

Table 1. Mean ± SE values of monthly maximum and minimum temperature (°C) and relative humidity (%) during the study period.

Months	Max. Temp (°C)	Min. Temp (°C)	Max. Relative Humidity (%)	Min. Relative Humidity (%)
January	28.55 ± 0.21	16.13 ± 0.16	78.71 ± 1.11	33.90 ± 1.44
February	31.25 ± 0.23	18.14 ± 0.28	64.82 ± 1.93	20.71 ± 0.62
April	35.40 ± 0.16	24.13 ± 0.35	63.43 ± 1.21	27.03 ± 1.16
May	34.03 ± 0.43	22.07 ± 0.24	81.07 ± 1.60	42.26 ± 2.65
July	31.43 ± 0.29	22.17 ± 0.25	84.20 ± 0.74	50.23 ± 1.43
August	28.13 ± 0.28	21.55 ± 0.15	86.07 ± 0.39	62.74 ± 1.81

Source: http/www.accuweather.com

Table 2. Mean ± SE values of monthly average temperature (°C) and relative humidity (%) during the study period.

Months	Monthly Average Temperature (°C)	Monthly Average Relative Humidity (%)
January	22.34 ± 0.16	56.31 ± 1.27
February	24.70 ± 0.25	42.77 ± 1.28
April	29.77 ± 0.25	45.23 ± 1.18
May	28.05 ± 0.33	61.66 ± 2.12
July	26.80 ± 0.27	67.22 ± 1.09
August	24.84 ± 0.21	74.40 ± 1.10

Table 3. Values of temperature humidity index (THI) during the study period.

Sl. No.	Months	Temperature Humidity Index (THI)	Seasons	Temperature Humidity Index (THI)
1	January	68.53	Winter	69.97 ± 1.14^{a}
2	February	70.86		
3	April	77.53	Summer	77.52 ± 0.12^{b}
4	May	77.50		
5	July	76.38	Rainy	75.29 ± 1.09^{b}
6	August	74.20		

Mean THI value with different superscripts (a and b) in the column differ significantly ($P<0.05$).

intermediate THI value during rainy months. The THI varied significantly ($P<0.5$) between the different seasons with summer (77.52 ± 0.12) and rainy season (75.29 ± 1.09) recording significantly higher ($P<0.05$) THI compared to the winter season (69.97 ± 1.14). But, THI of summer and rainy season did not differ significantly.

4.2 Plasma heat shock protein 70 levels (ng/mL)

The mean ± SE values of plasma HSP70 (ng/mL) in Hallikar cattle during study period of the year are depicted in the Table 4 and Fig. 1. Mean plasma HSP70 level was lowest (1.85 ± 0.06) during August and highest (3.54 ± 0.11) during July in control group. In supplemented group, lowest (1.94 ± 0.07) and highest (4.01 ± 0.22) levels were observed during the months of August and April, respectively.

In control and supplemented groups, plasma HSP70 levels recorded in the summer month of April and rainy month of July was significantly higher ($P<0.05$) than winter months of January and February and rainy month of August. In control group, significantly ($P<0.05$) lower levels were recorded during the month of August compared to the summer months and rainy month of July. In supplemented group, HSP70 levels during February and August months were significantly ($P<0.05$) lower compared to the summer months and rainy month of July. Plasma level of HSP70 was significantly ($P<0.05$) higher during the month of April in supplemented group compared to corresponding values in the control group.

The HSP70 concentration was significantly higher ($P<0.05$) during summer season compared to winter season in both control (3.08 ± 0.14 vs. 2.39 ± 0.09) and supplemented groups (3.52 ± 0.20 vs. 2.43 ± 0.18). But, there was no significant

(P>0.05) variation in HSP70 levels between control and supplement group in any of the season studied.

4.3 Erythrocyte antioxidant enzyme activities

4.3.1 Catalase activity (µmol of H_2O_2/min/g of Hb)

The values of erythrocyte catalase activity (µmol of H_2O_2/min/g of Hb) in Hallikar cattle during the study period are depicted in the Table 5 and Fig. 2. The lowest and the highest erythrocyte catalase activity recorded in the present study were 151.85 ± 3.85 and 195.35 ± 5.88 in control group and 127.31 ± 4.56 and 169.73 ± 3.49 in the supplemented group.

Erythrocyte catalase activity was significantly higher (P<0.5) during the summer month of April and May compared to winter months and rainy month of August in control group. In supplemented group, significantly (P<0.05) higher activity was noticed during the summer month of May compared to winter months and rainy month of August.

Catalase activity was significantly higher (P<0.05) during summer season, compared to winter and rainy season in both the groups. There was a significant decrease (P<0.05) in the catalase activity in supplemented group compared to control group during the summer and winter season but not during the rainy season.

4.3.2 Superoxide dismutase activity (units/g of Hb)

The values of erythrocyte superoxide dismutase (SOD) activity (units/g of Hb**)** in Hallikar cattle during the study period are presented in the Table 6 and Fig. 3.

Table 4. Mean ± SE values of plasma heat shock protein 70 (HSP70) levels (ng/mL) in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	2.43 ± 0.14^{abA}	2.49 ± 0.14^{abA}	Winter	2.39 ± 0.09^{aA}	2.43 ± 0.18^{aA}
February	2.35 ± 0.09^{abA}	2.36 ± 0.10^{aA}			
April	3.18 ± 0.22^{cdA}	4.01 ± 0.22^{dB}	Summer	3.08 ± 0.14^{bA}	3.52 ± 0.20^{bA}
May	2.98 ± 0.14^{bcdA}	3.04 ± 0.36^{bcA}			
July	3.54 ± 0.11^{dA}	3.53 ± 0.38^{cdA}	Rainy	2.70 ± 0.08^{abA}	2.74 ± 0.19^{aA}
August	1.85 ± 0.06^{aA}	1.94 ± 0.07^{aA}			

The values with different superscripts within a column (a, b, c and d) and within a row (A and B) differ significantly ($P<0.05$).

Table 5. Mean ± SE values of erythrocyte catalase activity (μmol of H_2O_2/min/g of Hb) in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	151.85 ± 3.85^{aA}	127.31 ± 4.56^{aB}	Winter	157.66 ± 3.07^{aA}	134.15 ± 5.48^{aB}
February	163.47 ± 3.41^{abA}	140.99 ± 8.69^{abB}			
April	182.23 ± 4.39^{cdA}	156.52 ± 2.93^{bcB}	Summer	188.79 ± 2.75^{bA}	163.13 ± 2.08^{cB}
May	195.35 ± 5.88^{dA}	169.73 ± 3.49^{cB}			
July	169.60 ± 7.81^{bcA}	154.57 ± 1.61^{bcA}	Rainy	161.18 ± 3.74^{aA}	150.45 ± 3.00^{bA}
August	152.75 ± 2.73^{aA}	146.33 ± 5.03^{bA}			

The values with different superscripts within a column (a, b, c and d) and within a row (A and B) differ significantly ($P<0.05$).

Fig. 1. Plasma levels of heat shock protein 70 (HSP70) in Hallikar cattle during the study period.

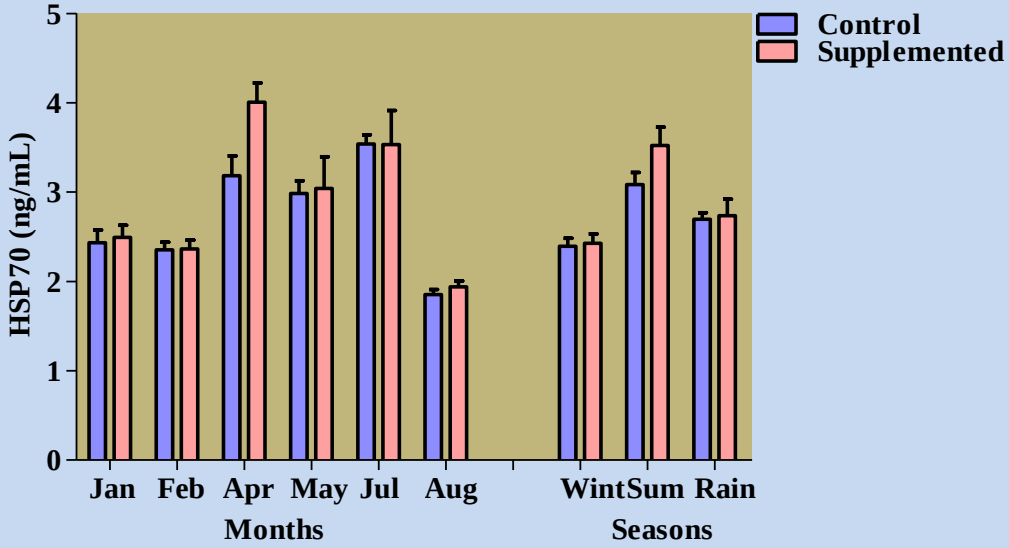
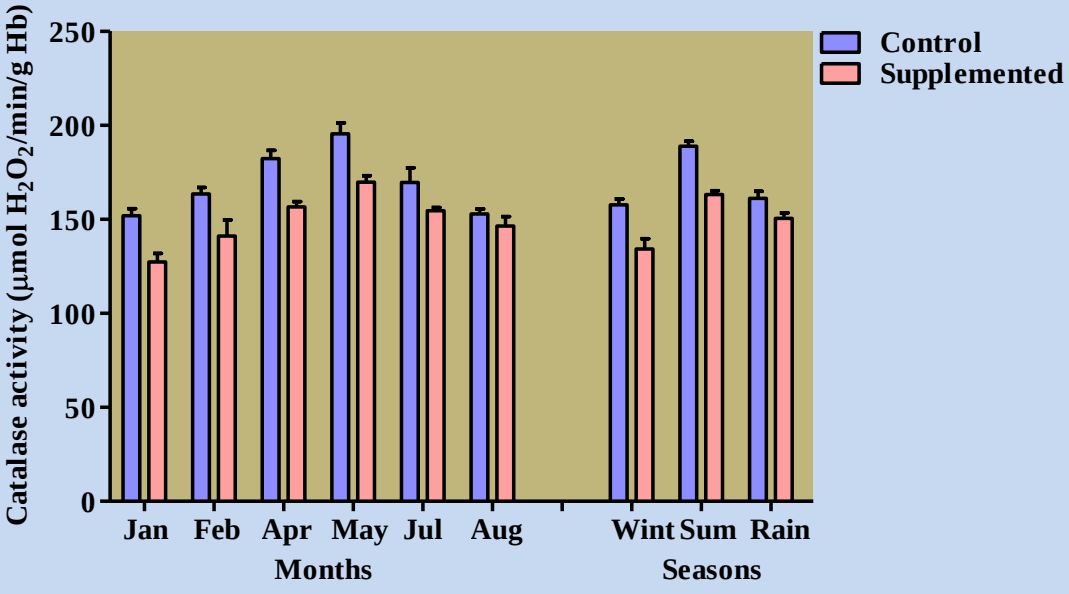


Fig. 2. Erythrocyte catalase activity in Hallikar cattle during the study period.



The erythrocyte superoxide dismutase activity (units/g Hb) ranged from 265.61 ± 8.15 to 364.56 ± 5.02 and 146.66 ± 6.68 to 207.71 ± 8.33 in control and supplemented group, respectively.

In control group, superoxide dismutase activity of summer months (April and May) was significantly higher ($P<0.05$) compared to winter months and rainy month of August. In supplemented group, significantly higher ($P<0.05$) superoxide dismutase activity was observed during the months of May compared to winter months of January and February. There was significant reduction ($P<0.05$) in the erythrocyte superoxide dismutase activity in supplemented group compared to control group during all months of the study.

Significantly higher ($P<0.05$) superoxide dismutase activity was recorded during summer season compared to winter and rainy seasons in control group. In supplemented group, activity was significantly higher ($P<0.05$) during summer season compared to winter and rainy season. Superoxide dismutase activity got reduced significantly ($P<0.05$) in supplemented group compared to control group during all the seasons.

4.3.3 Glutathione peroxidase activity (units/g of Hb)

The values of erythrocyte glutathione peroxidase activity in Hallikar cattle during the study period are depicted in Table 7 and Fig. 4. In the control and supplemented groups, the range of the erythrocyte glutathione peroxidase activity was 2672.66 ± 110.03 to 3430.30 ± 49.87 and 2435.93 ± 52.29 to 3024.71 ± 47.94, respectively.

Table 6. Mean ± SE values of erythrocyte superoxide dismutase activity (units/g of Hb) in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	276.38 ± 2.73^{aA}	146.66 ± 6.68^{aB}	Winter	296.00 ± 4.49^{aA}	157.26 ± 4.76^{aB}
February	315.61 ± 8.09^{bA}	167.86 ± 3.22^{abB}			
April	352.61 ± 6.39^{cA}	183.54 ± 2.61^{bcB}	Summer	358.58 ± 1.43^{bA}	195.62 ± 4.89^{bB}
May	364.56 ± 5.02^{cA}	207.71 ± 8.33^{cB}			
July	322.96 ± 17.85^{bcA}	191.84 ± 2.68^{bcB}	Rainy	299.28 ± 9.70^{aA}	187.58 ± 5.02^{bB}
August	265.61 ± 8.15^{aA}	183.32 ± 8.38^{bcB}			

The values with different superscripts within a column (a, b and c) and within a row (A and B) differ significantly ($P<0.05$).

Table 7. Mean ± SE values of erythrocyte glutathione peroxidase activity (units/g of Hb) in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	2882.27 ± 25.68^{abA}	2590.60 ± 64.57^{abB}	Winter	3031.25 ± 30.42^{bA}	2689.23 ± 83.30^{aB}
February	3180.23 ± 58.88^{cdA}	$2787.86 \pm 117.85^{bcdB}$			
April	3305.90 ± 46.40^{dA}	2920.81 ± 90.54^{cdB}	Summer	3368.10 ± 24.0^{cA}	2971.09 ± 44.92^{bB}
May	3430.30 ± 49.87^{dA}	3024.71 ± 47.94^{dB}			
July	3019.30 ± 98.20^{bcA}	2758.03 ± 55.98^{bcA}	Rainy	2845.98 ± 62.23^{aA}	2596.98 ± 37.45^{aB}
August	2672.66 ± 110.03^{aA}	2435.93 ± 52.29^{aA}			

The mean values with different superscripts within a column (a, b, c and d) and within a row (A and B) differ significantly ($P<0.05$).

Fig. 3. Erythrocyte superoxde dismutase activity in Hallikar cattle during the study period.

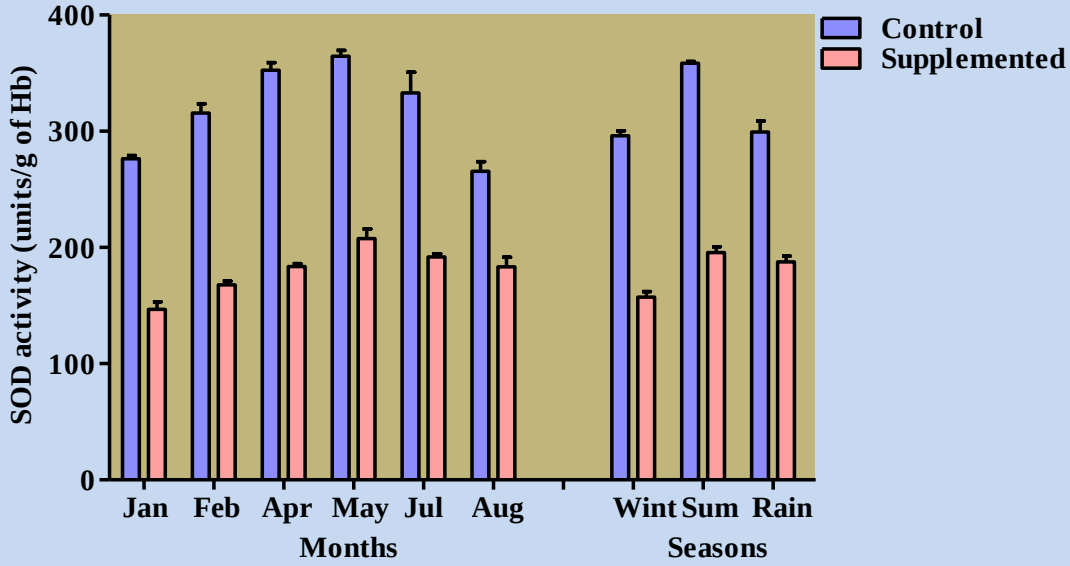
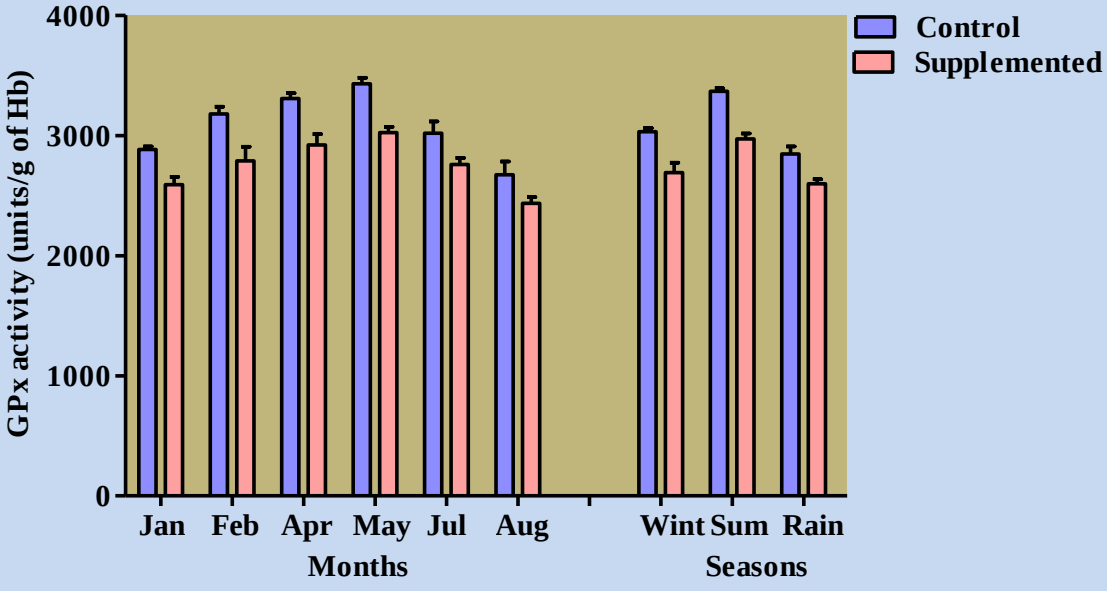


Fig. 4. Erythrocyte glutathione peroxidase (GPx) activity in Hallikar cattle during the study period.



Significantly higher (P<0.05) glutathione peroxidase activity was observed during the summer months of April and May compared to winter month of January and rainy months in control group. In the supplemented group, significantly higher (P<0.05) glutathione peroxidase activity was recorded during the summer months of April and May compared to winter month of January and rainy month of August. The enzyme activity varied significantly (P<0.05) between the control and supplemented groups during all the months of the study except July and August.

The activity of the glutathione peroxidase was significantly higher (P<0.05) during summer season compared to winter and rainy season in both the groups and the activity in the supplemented group showed significant reduction (P<0.05) compared to control group during all the seasons.

4.4 Serum biochemical profile

4.4.1 Serum enzyme profile

4.4.1.1 Serum aspartate aminotransferase (AST) activity (IU/L)

The values of serum aspartate aminotransferase activity in Hallikar cattle during different months of the study period are depicted in Table 8 and Fig. 5.

In the present study, the lowest and highest AST activity recorded in control and supplemented groups were 67.53 ± 1.78, 88.79 ± 3.13 and 59.22 ± 2.31, 74.53 ± 0.88, respectively.

The AST activity in the summer months of April and May was significantly higher (P<0.05) than winter months of January and February and rainy month of August

in control group. But, in the supplemented group, only the activity of May month was significantly higher ($P<0.05$) compared to all other months studied. The serum AST activity showed significant reduction ($P<0.05$) in supplemented group compared to control group during all months of the study except February.

In control group and supplemented group, AST activity was significantly higher ($P<0.05$) during summer season compared to other seasons and significantly lower ($P<0.05$) in the winter season compared to summer and rainy seasons. Significant reduction ($P<0.05$) in the AST activity in supplemented group compared to control group in all seasons was also observed in the present study.

4.4.1.2 Serum alanine aminotransferase (ALT) activity (IU/L)

The values of serum alanine aminotransferase activity in Hallikar cattle during the study period are depicted in Table 9 and Fig. 6. The lowest and the highest activity of serum alanine aminotransferase observed in the study were 37.27 ± 2.05, 74.85 ± 1.80 and 25.68 ± 1.50, 55.92 ± 4.41 in control and supplemented groups, respectively. In both control and supplemented groups, the serum ALT levels recorded during the summer month of May was significantly higher ($P<0.05$) compared to the levels recorded during all other months.

In control group, the ALT activity was significantly lower ($P<0.05$) during the rainy month of August compared to summer months and winter month of February and rainy month of July. In supplemented group, significantly lower ($P<0.05$) activity was observed during August compared to all other months studied.

Table 8. Mean ± SE values of serum aspartate aminotransferase (AST) activity (IU/L) in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	69.00 ± 2.31^{aA}	60.17 ± 2.57^{abB}	Winter	68.27 ± 1.35^{aA}	59.69 ± 1.92^{aB}
February	67.53 ± 1.78^{aA}	59.22 ± 2.31^{aA}			
April	88.79 ± 3.13^{dA}	64.10 ± 1.55^{abB}	Summer	85.23 ± 1.54^{cA}	69.32 ± 1.00^{cB}
May	87.67 ± 2.88^{dA}	74.53 ± 0.88^{cB}			
July	83.32 ± 1.79^{cdA}	67.12 ± 1.16^{bB}	Rainy	80.08 ± 1.02^{bA}	64.76 ± 1.31^{bB}
August	76.83 ± 1.87^{bcA}	62.40 ± 2.88^{abB}			

The values with different superscripts within a column (a, b, c and d) and within a row (A and B) differ significantly ($P<0.05$).

Table 9. Mean ± SE values of serum alanine aminotransferase (ALT) activity (IU/L) in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	43.65 ± 2.70^{abA}	33.50 ± 1.24^{bB}	Winter	44.55 ± 1.84^{aA}	35.03 ± 1.14^{aB}
February	45.45 ± 2.56^{bcA}	36.56 ± 1.48^{bB}			
April	51.23 ± 2.65^{cA}	37.60 ± 1.57^{bB}	Summer	63.04 ± 2.16^{bA}	46.78 ± 2.55^{bB}
May	74.85 ± 1.80^{dA}	55.92 ± 4.41^{cB}			
July	43.82 ± 1.59^{bcA}	35.40 ± 1.63^{bA}	Rainy	40.54 ± 1.17^{aA}	30.54 ± 1.01^{aB}
August	37.27 ± 2.05^{aA}	25.68 ± 1.50^{aB}			

The values with different superscripts within a column (a, b, c and d) and within a row (A and B) differ significantly ($P<0.05$).

Fig. 5. Serum aspartate aminotransferase (AST) activity in Hallikar cattle during the study period.

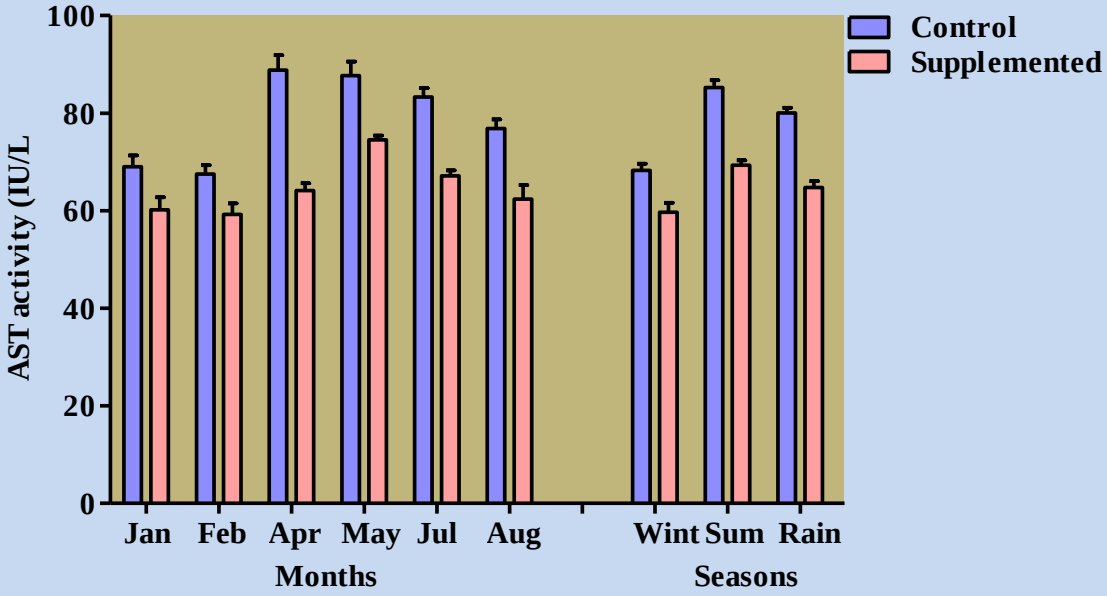
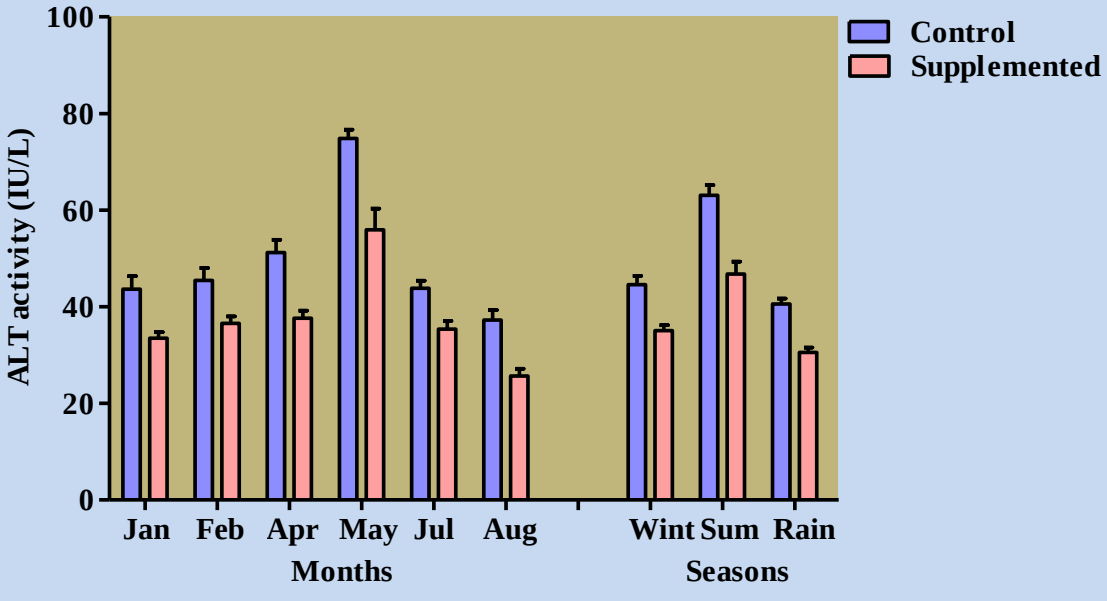


Fig. 6. Serum alanine aminotransferase (ALT) activity in Hallikar cattle during the study period.



In the summer season, the ALT activity was significantly higher ($P<0.05$) compared to winter and rainy season in both control and supplemented groups. Compared to control group, the activity was significantly declined ($P<0.05$) in supplemented group during all the seasons.

4.4.1.3 Serum alkaline phosphatase (ALP) activity (IU/L)

The values of serum alkaline phosphatase activity in Hallikar cattle during the study period are presented in Table 10 and Fig. 7. The range of mean serum alkaline phosphatase activity was 91.26 ± 1.28 to 140.41 ± 1.95 and 74.52 ± 1.76 to 93.31 ± 1.73 in control and supplemented groups, respectively.

Significantly ($P<0.05$) higher serum ALP activity was observed during summer month of April compared to the winter and rainy months in control group. But in supplemented group, ALP activity was significantly higher ($P<0.05$) during May compared to winter months of January and February and rainy month of August. In both groups, the enzyme activity of January month was significantly lower ($P<0.05$) than that of all other months of the study. There was a significant reduction ($P<0.05$) in ALP enzyme activity in the supplemented group compared to control group during all the months studied.

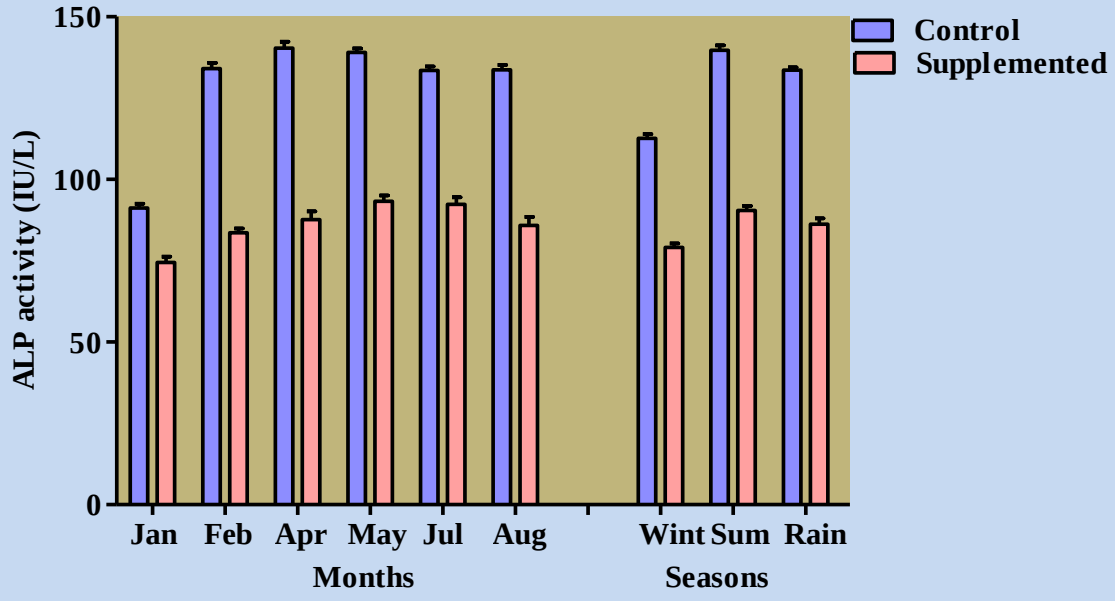
The serum ALP activity was significantly higher ($P<0.05$) during the summer season (139.75 ± 1.47) compared to winter (112.64 ± 1.32) and rainy season (133.57 ± 0.91) in control group. The enzyme activity during all the seasons was significantly lowered ($P<0.05$) in supplemented group than control group.

Table 10. Mean ± SE levels of serum alkaline phosphatase (ALP) activity (IU/L) in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	91.26 ± 1.28^{aA}	74.52 ± 1.76^{aB}	Winter	112.64 ± 1.32^{aA}	79.06 ± 1.24^{aB}
February	134.02 ± 1.79^{bA}	83.60 ± 1.34^{bB}			
April	140.41 ± 1.95^{cA}	87.67 ± 2.48^{bcdB}	Summer	139.75 ± 1.47^{cA}	90.49 ± 1.38^{bB}
May	139.09 ± 1.18^{bcA}	93.31 ± 1.73^{dB}			
July	133.48 ± 1.27^{bA}	92.32 ± 2.20^{cdB}	Rainy	133.57 ± 0.91^{bA}	86.22 ± 1.78^{bB}
August	133.66 ± 1.53^{bA}	85.89 ± 2.62^{bcB}			

The values with different superscripts within a column (a, b, c and d) and within a row (A and B) differ significantly ($P<0.05$).

Fig. 7. Serum alkaline phosphatase (ALP) activity in Hallikar cattle during the study period.



4.4.2 Serum protein profile

4.4.2.1 Serum total protein (g/dL)

The values of serum total protein level in Hallikar cattle during the study period of the year are presented in Table 11 and Fig. 8. The lowest mean serum total protein level was recorded during May month in control (6.00 ± 0.18) and July month in supplemented group (6.97 ± 0.31) and highest concentration during February (7.23 ± 0.10) and April (8.17 ± 0.22) months in control and supplemented group, respectively.

The level of the total protein was significantly higher ($P<0.05$) during all the months during the study except May in control group. However, in supplemented group, its level was significantly higher ($P<0.05$) during the summer months of April and May and rainy month of August compared to rainy month of July. The serum total protein level was significantly lower ($P<0.05$) in the control group compared to supplemented group during all the months of study.

There was significant reduction ($P<0.05$) in the levels of the serum total protein during summer season compared to winter season in control group. Further, the levels of serum total protein was significantly higher ($P<0.05$) in supplemented group compared to control group.

4.4.2.2 Serum albumin (g/dL)

The values of serum albumin (g/dL) level in Hallikar cattle during the study period are presented in Table 12 and Fig. 9. In control group, mean serum albumin level

Table 11. Mean ± SE values of serum total protein (g/dL) level in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	7.00 ± 0.11^{bA}	7.62 ± 0.13^{abB}	Winter	7.12 ± 0.08^{bA}	7.63 ± 0.08^{aB}
February	7.23 ± 0.10^{bA}	7.65 ± 0.09^{abB}			
April	6.88 ± 0.37^{bA}	8.17 ± 0.22^{bB}	Summer	6.44 ± 0.18^{aA}	8.08 ± 0.17^{bB}
May	6.00 ± 0.18^{aA}	8.00 ± 0.34^{bB}			
July	6.80 ± 0.07^{bA}	6.97 ± 0.31^{aB}	Rainy	6.78 ± 0.07^{aA}	7.52 ± 0.17^{aB}
August	6.77 ± 0.09^{bA}	8.07 ± 0.19^{bB}			

The values with different superscripts within a column (a and b) and within a row (A and B) differ significantly ($P<0.05$).

Table 12. Mean ± SE values of serum albumin level (g/dL) in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	3.37 ± 0.21^{abA}	3.65 ± 0.20^{abA}	Winter	3.58 ± 0.10^{aA}	3.60 ± 0.12^{aA}
February	3.80 ± 0.10^{bA}	3.55 ± 0.12^{bA}			
April	3.27 ± 0.20^{aA}	3.87 ± 0.13^{abA}	Summer	3.38 ± 0.14^{aA}	3.78 ± 0.08^{aA}
May	3.48 ± 0.14^{abA}	3.70 ± 0.20^{abA}			
July	3.38 ± 0.21^{abA}	3.85 ± 0.11^{aA}	Rainy	3.62 ± 0.14^{aA}	3.88 ± 0.13^{aA}
August	3.85 ± 0.17^{bA}	3.92 ± 0.18^{abA}			

The values with different superscripts within a column (a and b) and within a row (A and B) differ significantly ($P<0.05$).

Fig. 8. Serum total protein levels in Hallikar cattle during the study period.

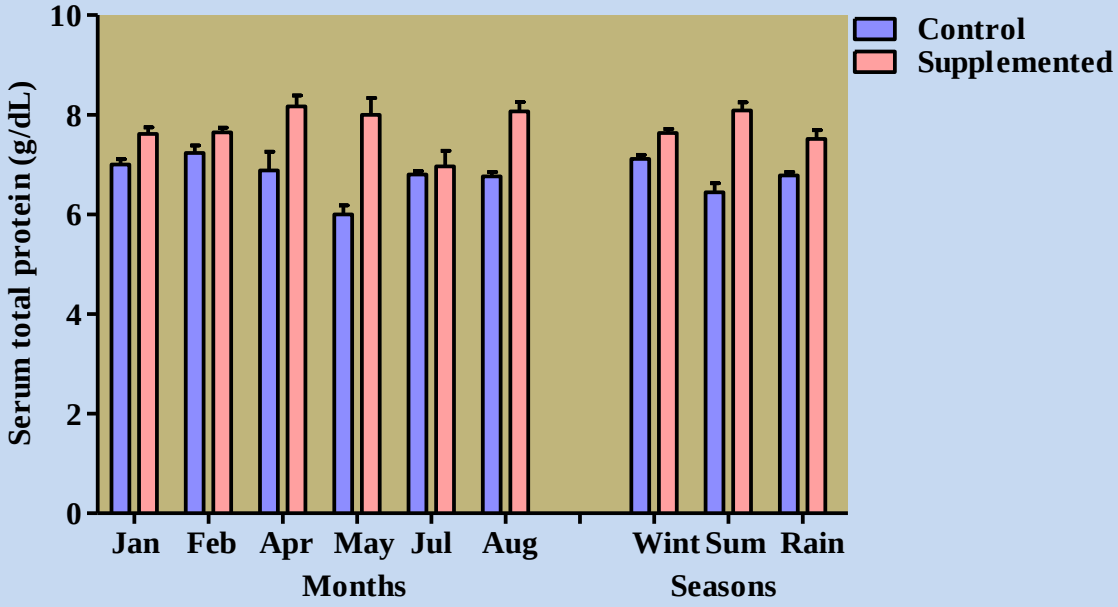
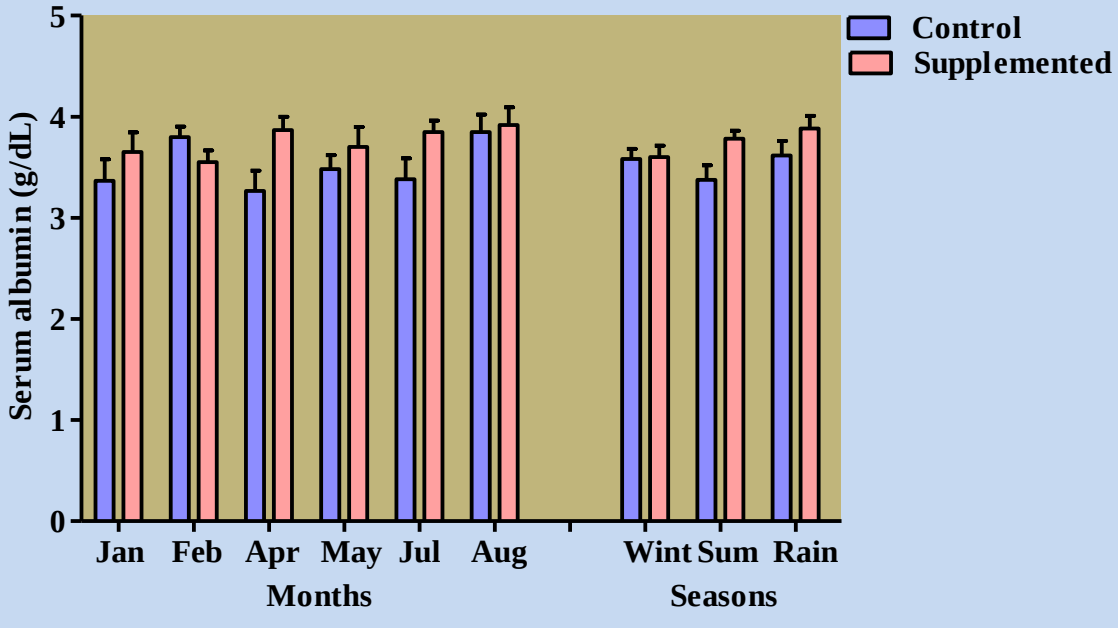


Fig. 9. Serum albumin levels in Hallikar cattle during the study period.



(g/dL) varied from 3.27 ± 0.20 to 3.85 ± 0.17 and in the supplemented group level ranged from 3.55 ± 0.12 to 3.92 ± 0.18.

Summer months of April showed significantly lower ($P<0.05$) serum albumin level compared to winter month of February and rainy month of August in control group. In supplemented group, level of serum albumin was significantly lower ($P<0.05$) in July compared to winter month of February. The serum albumin level did not vary significantly ($P>0.05$) between control and supplemented group during the entire period of the study.

In both control and supplemented group, no significant ($P>0.05$) variation in the serum albumin level was found among the seasons and serum albumin level did not vary significantly ($P>0.05$) between the control and supplemented groups during any of the seasons.

4.4.2.3 Serum globulin (g/dL)

The values of serum globulin level in Hallikar cattle during the study period are presented in Table 13 and Fig. 10. The lowest and highest level of mean serum globulin was 2.52 ± 0.18 to 3.63 ± 0.28 and 3.12 ± 0.22 to 4.30 ± 0.49 in control and supplemented groups, respectively.

Significantly lower ($P<0.05$) serum globulin level was recorded during summer month of May compared to summer month of April and winter months and rainy month of July in control group. In supplemented group, the level was significantly higher ($P<0.05$) during the summer months, winter month of February and rainy month of

August. The serum globulin level did not vary significantly (P>0.05) between control and supplemented groups during all the months of the study period except May and August.

The serum globulin level did not vary significantly (P>0.05) between the seasons in control group. Supplemented group possessed significantly higher (P<0.05) serum globulin levels compared to control group only during summer season.

4.4.3 Serum lipid profile

4.4.3.1 Serum triglycerides (mg/dL)

The values of serum triglycerides level in Hallikar cattle during the study period are presented in Table 14 and Fig. 11. The serum triglycerides level in the present study ranged from 22.15 ± 2.11 to 31.77 ± 1.73 in control group and 30.12 ± 0.60 to 38.97 ± 1.12 in supplemented group.

Serum triglyceride concentrations during summer months, winter month of February and rainy month of July were significantly lower (P<0.05) compared that of January in control group. In the supplemented group, serum triglyceride level was significantly lower (P<0.05) in the rainy month of July and summer month of May compared to winter month of January and rainy month of August.

In control group, level of serum triglyceride recorded during summer season was significantly lower (P<0.05) than the winter month. Further, the triglyceride levels in the supplemented group during all the three seasons were significantly higher (P<0.05) compared to the corresponding values of the control group.

Table 13. Mean ± SE values of serum globulin level (g/dL) in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	3.63 ± 0.28^{bA}	3.97 ± 0.31^{abA}	Winter	3.53 ± 0.17^{aA}	4.03 ± 0.18^{abA}
February	3.43 ± 0.17^{bA}	4.10 ± 0.19^{bA}			
April	3.62 ± 0.42^{bA}	4.30 ± 0.15^{bA}	Summer	3.07 ± 0.24^{aA}	4.30 ± 0.24^{bB}
May	2.52 ± 0.18^{aA}	4.30 ± 0.49^{bB}			
July	3.42 ± 0.23^{bA}	3.12 ± 0.22^{aA}	Rainy	3.17 ± 0.16^{aA}	3.63 ± 0.08^{aA}
August	2.92 ± 0.17^{abA}	4.15 ± 0.23^{bB}			

The values with different superscripts within a column (a and b) and within a row (A and B) differ significantly ($P<0.05$).

Table 14. Mean ± SE values of serum triglycerides (mg/dL) level in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	31.77 ± 1.73^{bA}	38.97 ± 1.12^{cB}	Winter	27.59 ± 1.09^{bA}	35.93 ± 1.15^{aB}
February	23.42 ± 0.69^{aA}	32.90 ± 2.44^{abB}			
April	22.15 ± 2.11^{aA}	32.97 ± 2.30^{abB}	Summer	22.41 ± 1.65^{aA}	32.35 ± 1.22^{aB}
May	22.67 ± 1.87^{aA}	31.73 ± 1.88^{aB}			
July	23.58 ± 0.77^{aA}	30.12 ± 0.60^{aB}	Rainy	25.44 ± 0.81^{abA}	33.68 ± 1.11^{aB}
August	27.30 ± 1.28^{abA}	37.23 ± 2.04^{bcB}			

The values with different superscripts within a column (a, b and c) and within a row (A and B) differ significantly ($P<0.05$).

Fig. 10. Serum globulin levels in Hallikar cattle during the study period.

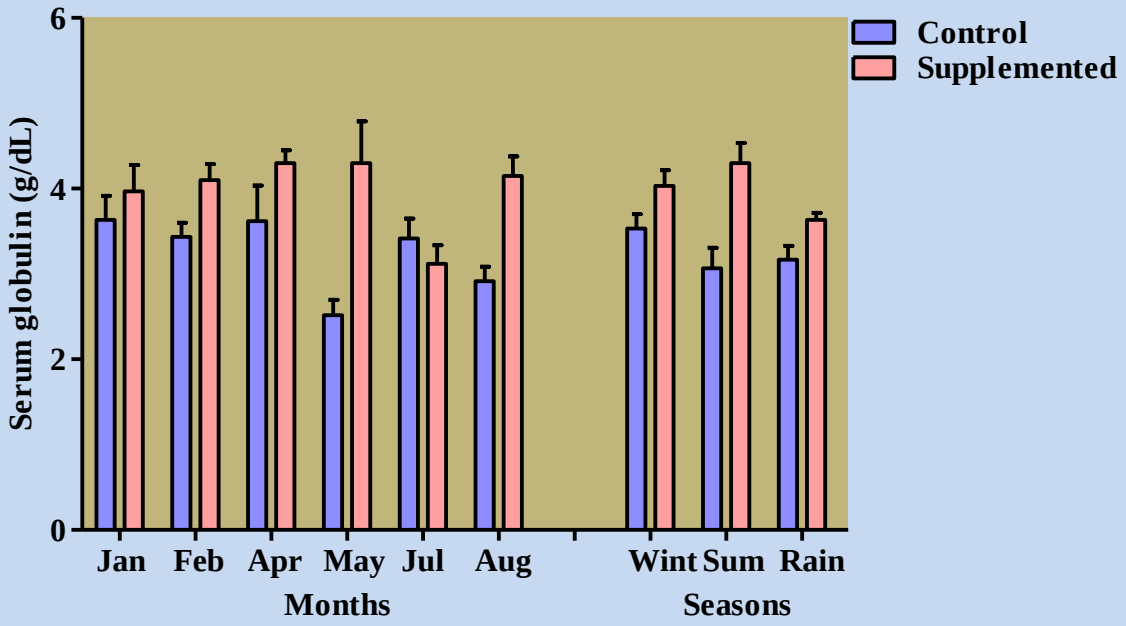
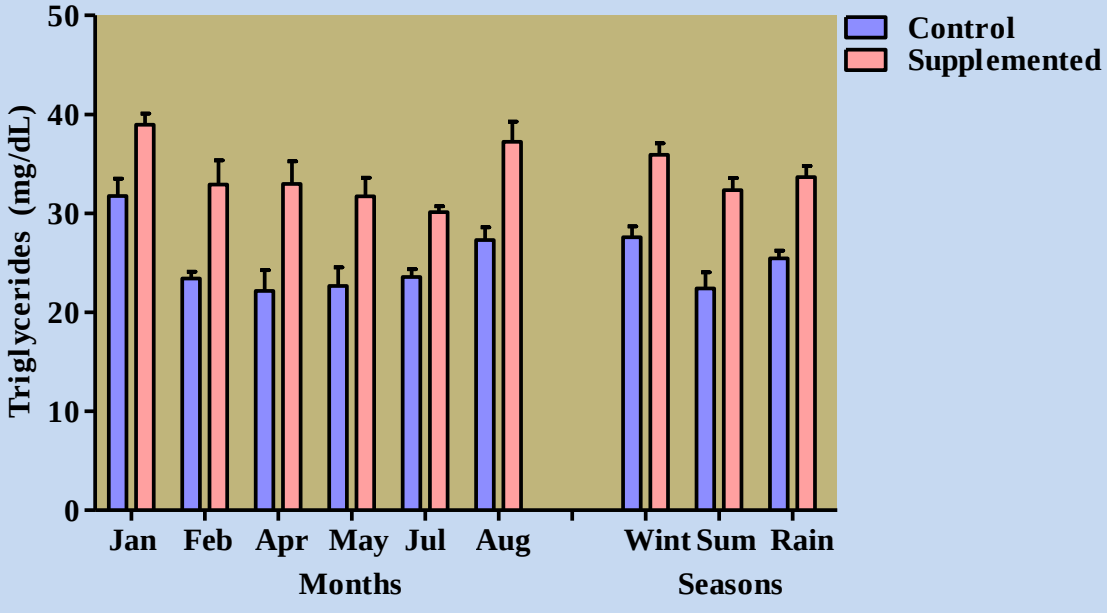


Fig. 11. Serum levels of triglycerides in Hallikar cattle during the study period.



4.4.3.2 Serum total cholesterol (mg/dL)

The values of serum total cholesterol levels in Hallikar cattle during the study period are depicted in Table 15 and Fig. 12. In the present study, serum total cholesterol level in control and supplemented group varied from 113.17 ± 5.26 to 196.17 ± 4.93 and 155.00 ± 7.74 to 266.67 ± 5.16, respectively.

Compared to all other months, serum total cholesterol level during summer month of May and rainy month of July was significantly higher ($P<0.05$) in both control and supplemented groups. In control group, the cholesterol level was significantly lower ($P<0.05$) during the winter months of January and February compared to summer and rainy months and in supplemented group, significantly lower ($P<0.05$) level was observed during the winter month of February compared to summer months and rainy month of July. The serum level of total cholesterol was significantly higher ($P<0.05$) in supplemented group compared to control group during all months of the study.

Serum level of total cholesterol was significantly higher ($P<0.05$) during summer compared to winter season in control group. Further, the supplemented group showed significant increment ($P<0.05$) in cholesterol levels compared to control group during all the seasons.

4.4.3.3 Serum high density lipoprotein-cholesterol (HDL-C) (mg/dl)

The values of serum HDL-C levels in Hallikar cattle during the study period are depicted in Table 16 and Fig. 13. The range of serum HDL cholesterol levels

Table 15. Mean ± SE values of serum level of total cholesterol (mg/dL) in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	113.17 ± 5.26^{aA}	169.67 ± 4.26^{abB}	Winter	117.92 ± 4.07^{aA}	162.33 ± 5.15^{aB}
February	122.67 ± 8.49^{aA}	155.00 ± 7.74^{aB}			
April	150.50 ± 8.09^{bA}	182.00 ± 6.85^{bB}	Summer	168.25 ± 5.63^{bA}	200.17 ± 4.50^{bB}
May	186.00 ± 6.32^{cA}	218.33 ± 6.11^{cB}			
July	196.17 ± 4.93^{cA}	266.67 ± 5.16^{dB}	Rainy	173.00 ± 5.60^{bA}	222.58 ± 6.18^{cB}
August	149.83 ± 8.56^{bA}	178.50 ± 11.15^{abB}			

The values with different superscripts within a column (a, b, c and d) and within a row (A and B) differ significantly ($P<0.05$).

Table 16. Mean ± SE values of serum HDL cholesterol (mg/dL) level in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	8.53 ± 0.32^{aA}	12.02 ± 0.29^{aB}	Winter	8.01 ± 0.51^{aA}	11.38 ± 0.28^{aB}
February	7.48 ± 1.10^{aA}	10.75 ± 0.52^{aB}			
April	7.12 ± 0.34^{aA}	14.57 ± 1.62^{bcdB}	Summer	7.16 ± 0.23^{aA}	14.70 ± 1.07^{bcB}
May	7.20 ± 0.23^{aA}	14.83 ± 0.58^{cdB}			
July	8.77 ± 0.39^{aA}	14.93 ± 0.44^{dB}	Rainy	8.19 ± 0.23^{aA}	12.88 ± 0.36^{acB}
August	7.62 ± 0.58^{aA}	10.82 ± 0.43^{aB}			

The values with different superscripts within a column (a, b, c and d) and within a row (A and B) differ significantly ($P<0.05$).

Fig. 12. Serum total cholesterol levels in Hallikar cattle during the study period.

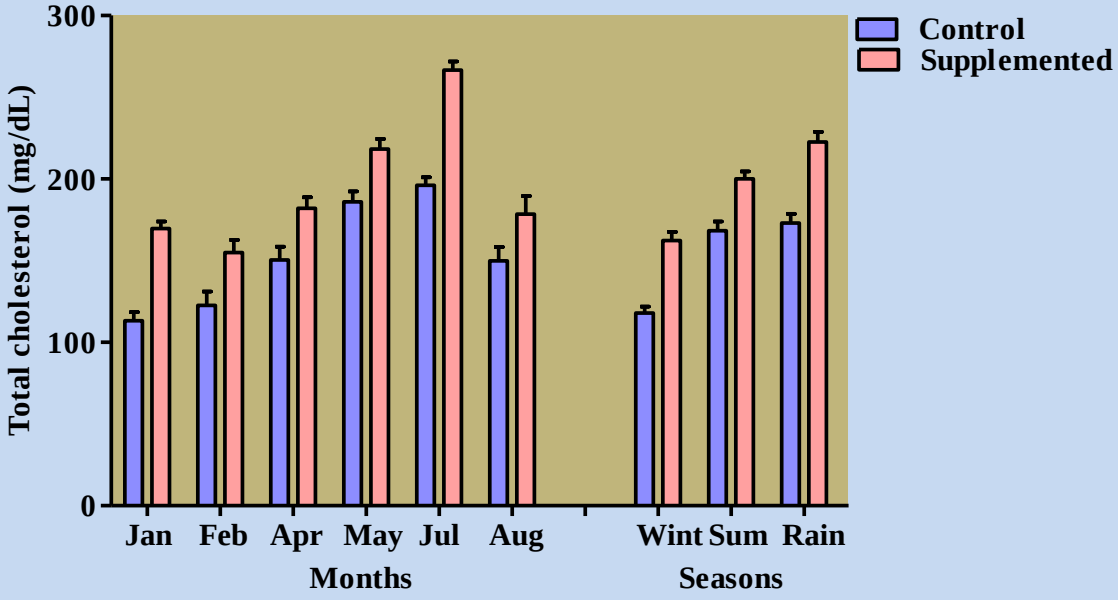
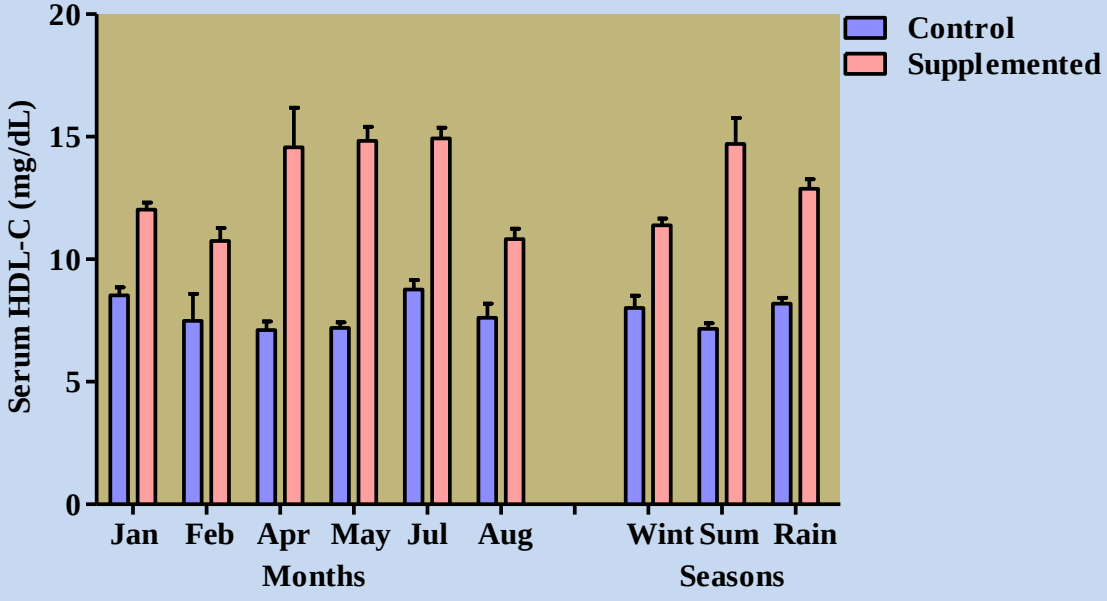


Fig. 13. Serum HDL cholesterol levels in Hallikar cattle during the study period.



in control and supplemented groups was of 7.12 ± 0.34 to 8.77 ± 0.39 and 10.75 ± 0.52 to 14.93 ± 0.44, respectively.

In control group, the HDL-C level did not vary significantly (P>0.05) among the different months. But, in supplemented group, HDL-C levels during the summer months of April, May and rainy month of July were significantly higher (P<0.05) than that of the other months. There was a significant elevation (P<0.05) in the serum HDL-C levels in supplemented group compared to control group during all the months studied.

In control group, HDL cholesterol level did not show any significant (P>0.05) variation among the seasons But, it was significantly higher (P<0.05) during summer compared to winter season in supplemented group. HDL cholesterol levels were significantly higher (P<0.05) in supplemented group compared to control group during all the seasons.

4.4.3.4 Serum low density lipoprotein cholesterol (LDL-C) (mg/dl)

The values of serum LDL cholesterol level in Hallikar cattle during the study period are presented in Table 17 and Fig. 14. The range of values for serum LDL cholesterol for control group was 98.28 ± 5.22 to 184.43 ± 4.66 and for supplemented group was 133.17 ± 10.91 to 248.21 ± 5.59.

In the control group, significantly higher (P<0.05) levels of serum LDL-C was observed during the summer and rainy months compared to the winter months. But in supplemented group, LDL-C level was significantly higher (P<0.05) during summer month of May and rainy month of July compared to all other months. Significant increase

($P<0.05$) in the serum LDL-C level in supplemented group was observed only during May and July months.

Significantly higher ($P<0.05$) level of LDL-C was recorded during summer and rainy seasons compared to winter season in control group. Summer season LDL-C level did not vary significantly ($P>0.05$) between control and supplemented group. But, during winter and rainy season the LDL-C level increased significantly ($P<0.05$) in supplemented group compared to the corresponding values of control group.

4.4.3.5 Serum very low density lipoprotein cholesterol (VLDL-C) (mg/dL)

The mean ± SE values of serum VLDL-cholesterol in Hallikar cattle during the study period are presented in Table 18 and Fig. 15. Serum VLDL cholesterol level (mg/dL) ranged from 4.43 ± 0.42 to 6.35 ± 0.35 and 6.02 ± 0.12 to 7.93 ± 0.61 in control and supplemented groups, respectively.

In control group, the VLDL-C level was significantly lower ($P<0.05$) during summer months, winter month of February and rainy month of July compared winter month of January. However, in supplemented group, level of VLDL-C was significantly lower ($P<0.05$) during the summer month of May and rainy month of July compared winter month of January, summer month of April and rainy month of August. In the present study, significant ($P<0.05$) variation in the serum VLDL-C level was observed between the control and supplemented group during all the months of the study except during July.

Table 17. Mean ± SE values of serum LDL cholesterol (mg/dL) level in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	98.28 ± 5.22^{aA}	147.17 ± 4.22^{aB}	Winter	104.39 ± 4.40^{aA}	142.42 ± 4.54^{aB}
February	110.50 ± 8.96^{aA}	137.67 ± 7.89^{aA}			
April	138.95 ± 7.60^{bA}	133.17 ± 10.91^{aA}	Summer	161.69 ± 5.43^{bA}	165.16 ± 5.05^{bA}
May	184.43 ± 4.66^{cA}	197.15 ± 6.51^{bA}			
July	182.68 ± 4.57^{cA}	248.21 ± 5.59^{cB}	Rainy	159.72 ± 5.40^{bA}	192.47 ± 2.66^{cB}
August	136.76 ± 8.19^{bA}	136.74 ± 8.06^{aA}			

The values with different superscripts within a column (a, b and c) and within a row (A and B) differ significantly ($P<0.05$).

Table 18. Mean ± SE values of serum VLDL cholesterol (mg/dL) level in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	6.35 ± 0.35^{bA}	7.79 ± 0.22^{cB}	Winter	5.52 ± 0.22^{bA}	7.19 ± 0.23^{aB}
February	4.68 ± 0.14^{aA}	6.58 ± 0.49^{abB}			
April	4.43 ± 0.42^{aA}	7.93 ± 0.61^{cB}	Summer	4.48 ± 0.33^{aA}	7.14 ± 0.33^{aB}
May	4.53 ± 0.38^{aA}	6.35 ± 0.38^{aB}			
July	4.72 ± 0.15^{aA}	6.02 ± 0.12^{aA}	Rainy	5.09 ± 0.61^{abA}	6.74 ± 0.22^{aB}
August	5.46 ± 0.26^{abA}	7.45 ± 0.41^{bcB}			

The values with different superscripts within a column (a, b and c) and within a row (A and B) differ significantly ($P<0.05$).

Fig. 14. Serum LDL cholesterol levels in Hallikar cattle during the study period.

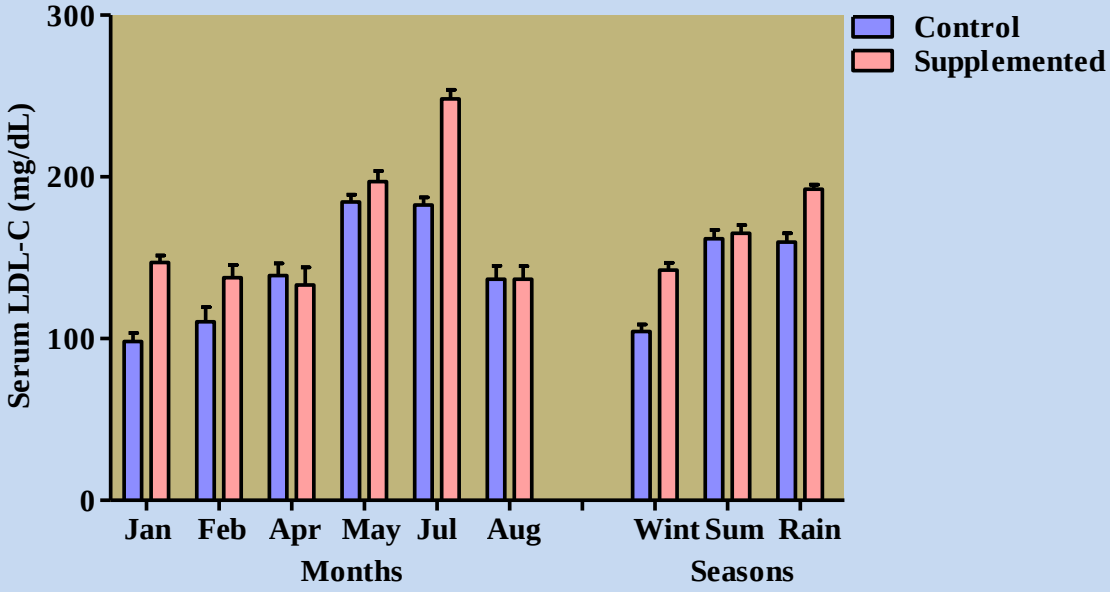
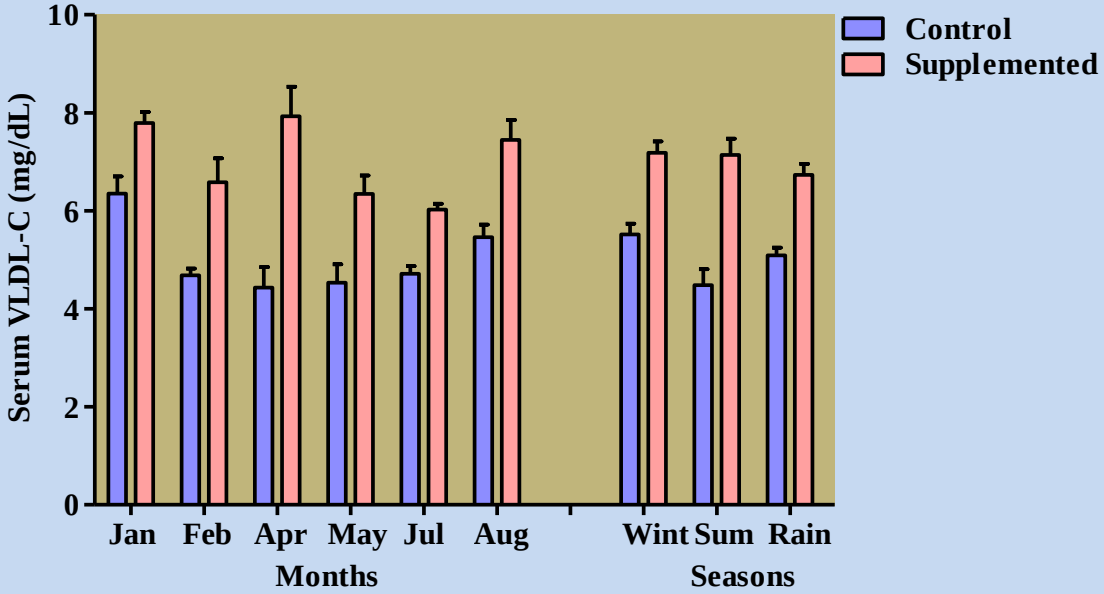


Fig. 15. Serum VLDL cholesterol levels in Hallikar cattle during the study period.



In the control group, it was significantly lower ($P<0.05$) during summer season compared to winter seasons however, serum VLDL-C levels did not vary significantly ($P>0.05$) during different seasons in the supplemented group. During all the seasons, supplemented group showed significantly higher ($P<0.05$) VLDL-C levels compared to control group.

4.4.4 Blood glucose (mg/dL)

The values of blood glucose level in Hallikar cattle during selected months of the year are depicted in Table 19 and Fig. 16. The mean blood glucose level ranged from 51.50 ± 1.54 to 61.83 ± 2.88 and 41.67 ± 1.67 to 51.33 ± 1.05 in control and supplemented groups, respectively.

In the control group, significantly higher ($P<0.05$) level of blood glucose was recorded during the summer month of April and May compared to the winter month of January and rainy month of August. In the supplemented group, significantly higher ($P<0.05$) level was recorded during summer month of May compared to January and August. Blood glucose level varied significantly ($P<0.05$) between the control and supplemented group during all months of the study.

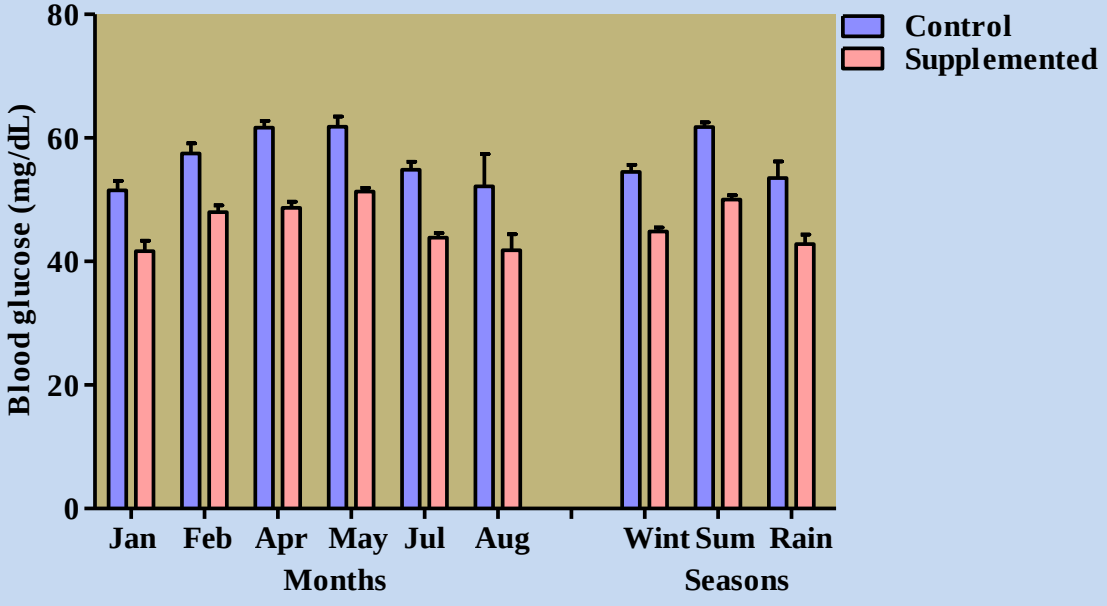
In control and supplemented group, the blood glucose levels increased significantly ($P<0.05$) during summer season compared to winter and rainy seasons and the supplemented group showed significantly lower ($P<0.05$) glucose levels compared to control group during all three seasons.

Table 19. Mean ± SE values of blood glucose level (mg/dL) in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	51.50 ± 1.54^{aA}	41.67 ± 1.67^{aB}	Winter	54.50 ± 1.17^{aA}	44.83 ± 0.67^{aB}
February	57.50 ± 1.61^{abA}	48.00 ± 1.07^{abB}			
April	61.67 ± 2.06^{bA}	48.67 ± 1.48^{abB}	Summer	61.75 ± 0.76^{bA}	50.00 ± 0.73^{bB}
May	61.83 ± 2.88^{bA}	51.33 ± 1.05^{bB}			
July	54.83 ± 1.30^{abA}	43.83 ± 0.79^{abB}	Rainy	53.50 ± 2.72^{aA}	42.83 ± 1.53^{aB}
August	52.17 ± 5.24^{aA}	41.83 ± 2.59^{aB}			

The values with different superscripts within a column (a, b, c and d) and within a row (A and B) differ significantly ($P<0.05$).

Fig. 16. Blood glucose levels in Hallikar cattle during the study period.



4.4.5 Plasma hormone profile

4.4.5.1 Plasma triiodothyronine (ng/mL)

The values of plasma triiodothyronine level in Hallikar cattle during the selected study months of the year are depicted in Table 20 and Fig. 17. The mean plasma triiodothyronine level ranged from 1.25 ± 0.07 to 1.74 ± 0.07 and 0.83 ± 0.06 to 1.24 ± 0.12 in control and supplemented groups, respectively.

In the present study, significantly lower ($P<0.05$) concentration of plasma triiodothyronine was recorded during the summer months of April and May compared to winter month of February and rainy month of August in control group. But, in the supplemented group, significantly lower ($P<0.05$) concentration was recorded during the summer months of April and May, winter month of January and rainy month of August compared to the winter month of February. Plasma triiodothyronine level was significantly lower ($P<0.05$) in supplemented compared to control group during all months of the study.

The plasma triiodothyronine level was significantly lower ($P<0.05$) during summer season compared to winter season in control group, however, it did not vary significantly ($P<0.05$) in the supplemented group. Further, level decreased significantly ($P<0.05$) in supplemented group compared to control group during all the seasons.

4.4.5.2 Plasma thyroxine (ng/mL)

The plasma thyroxine level in Hallikar cattle during selected study months of the year are depicted in Table 21 and Fig. 18.

Table 20. Mean ± SE values of plasma triiodothyronine (ng/mL) level in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	1.44 ± 0.15^{abcA}	0.87 ± 0.04^{aB}	Winter	1.59 ± 0.07^{bA}	1.05 ± 0.06^{aB}
February	1.74 ± 0.07^{cA}	1.24 ± 0.12^{bB}			
April	1.25 ± 0.07^{aA}	0.83 ± 0.06^{aB}	Summer	1.28 ± 0.06^{aA}	0.84 ± 0.03^{aB}
May	1.32 ± 0.11^{aA}	0.85 ± 0.07^{aB}			
July	1.43 ± 0.12^{abcA}	0.93 ± 0.50^{abB}	Rainy	1.52 ± 0.14^{abA}	0.89 ± 0.02^{aB}
August	1.62 ± 0.20^{bcA}	0.86 ± 0.03^{aB}			

The values with different superscripts within a column (a, b and c) and within a row (A and B) differ significantly ($P<0.05$).

Table 21. Mean ± SE values of plasma thyroxine (ng/mL) level in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	7.40 ± 0.67^{aA}	5.64 ± 0.65^{abA}	Winter	7.80 ± 0.48^{bcA}	5.56 ± 0.40^{aB}
February	8.20 ± 0.38^{abA}	5.48 ± 0.29^{aB}			
April	6.82 ± 0.24^{aA}	10.21 ± 0.21^{cB}	Summer	6.89 ± 0.44^{aA}	10.81 ± 0.55^{cB}
May	6.97 ± 0.77^{aA}	11.40 ± 0.98^{cB}			
July	8.01 ± 0.51^{abcA}	7.59 ± 0.85^{bA}	Rainy	9.12 ± 0.45^{cA}	8.48 ± 0.45^{bA}
August	10.24 ± 0.61^{cA}	7.28 ± 0.68^{abB}			

The values with different superscripts within a column (a, b and c) and within a row (A and B) differ significantly ($P<0.05$).

Fig. 17. Plasma levels of triiodothyronine in Hallikar cattle during the study period.

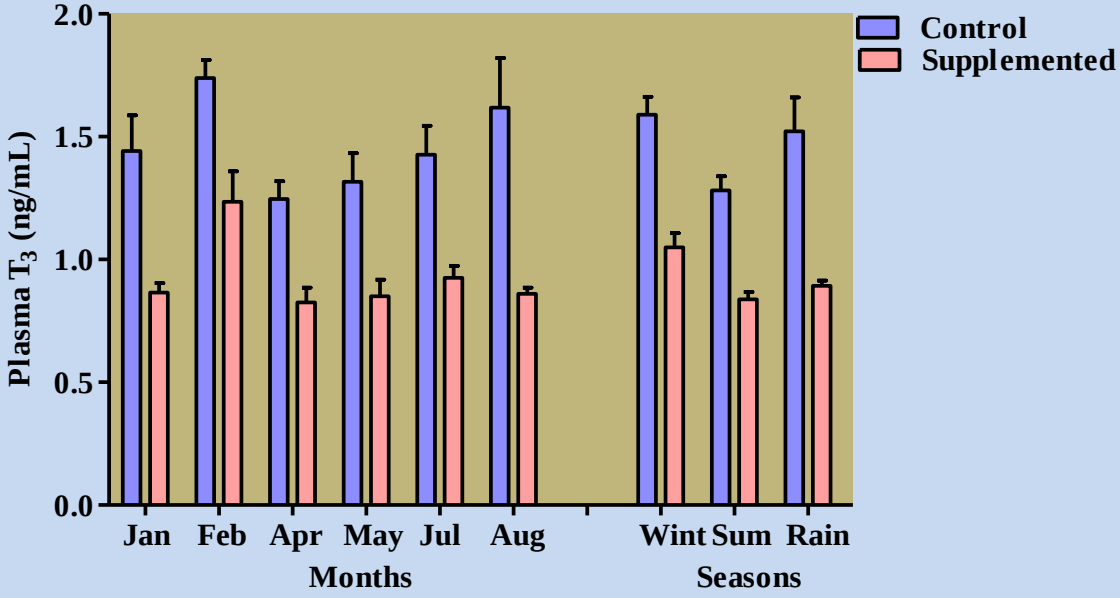
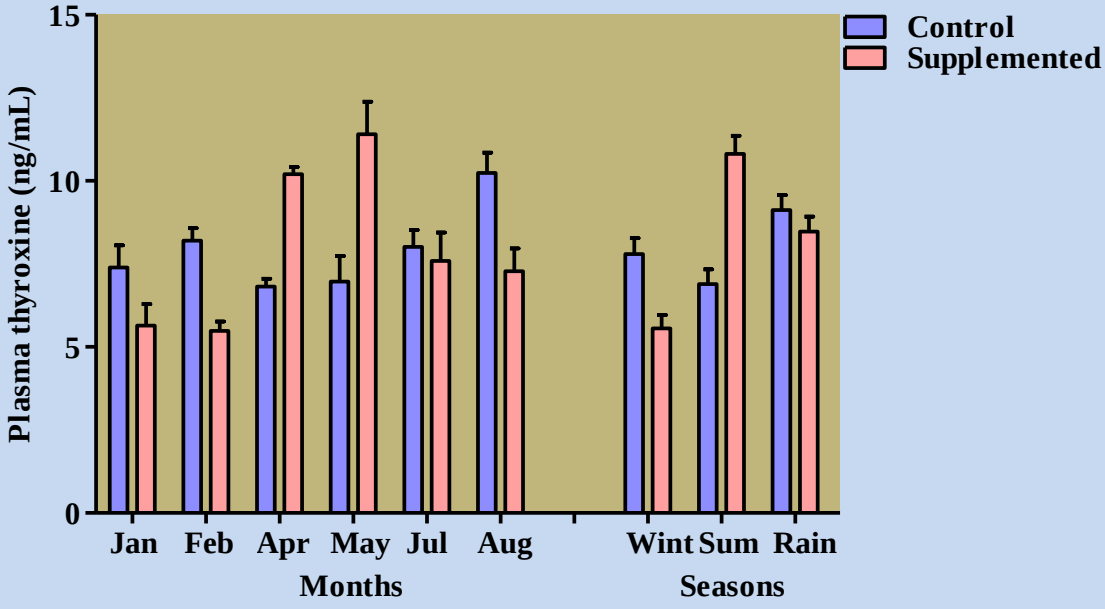


Fig. 18. Plasma levels of thyroxine in Hallikar cattle during the study period



The mean plasma thyroxine level ranged from 6.82 ± 0.24 to 10.24 ± 0.61 and 5.48 ± 0.29 to 11.40 ± 0.98 in the control and supplemented groups, respectively.

Significantly lower (P<0.05) levels of plasma thyroxine was recorded during the summer months of April and May and winter month of January compared to the rainy month of August in control group. In supplemented group, significantly lower (P<0.05) level of thyroxine was observed during winter month of February compared to summer months and rainy month of July. The plasma thyroxine level varied significantly (P<0.05) in supplemented group compared to control group during all months of the study except January and July.

The summer levels of thyroxine were significantly lower (P<0.05) compared to corresponding values of winter and rainy season in control group. In supplemented group the thyroxine levels varied significantly (P<0.05) from control group levels in winter all seasons except rainy season.

4.4.5.3 Plasma cortisol (nmol/L)

The values of plasma cortisol level in Hallikar cattle during selected months of the year are presented in Table 22 and Fig. 19. The plasma cortisol level in the present study ranged from 41.10 ± 2.43 to 57.89 ± 3.28 and 34.70 ± 1.31 to 48.76 ± 1.14 in control and supplemented group animals, respectively.

Significantly higher (P<0.05) plasma cortisol level was recorded during the summer month of April and May compared to rainy month of August in control group. In supplemented group, significantly higher (P<0.05) plasma cortisol was recorded during

the summer month of April compared to rainy months of July and August, winter month of January and summer month of May. There was significant reduction ($P<0.05$) in the plasma cortisol level in the supplemented group compared to control group only during the summer months of April and May.

The plasma cortisol level was significantly higher ($P<0.05$) during summer (53.64 ± 1.98) compared to winter (45.79 ± 1.09) and rainy seasons (42.09 ± 2.37) in control group. The values in the supplemented group were significantly lower ($P<0.05$) compared to that of control group during all the seasons.

4.4.5.4 Plasma insulin (μIU/mL)

The values of plasma insulin level in Hallikar cattle during the selected study months of the year are presented in Table 23 and Fig. 20. The plasma insulin level in the present study ranged from 14.82 ± 1.97 to 22.97 ± 2.31 and 24.95 ± 3.05 to 38.34 ± 2.07 in control and supplemented group animals, respectively.

In control group, the plasma insulin level varied significantly ($P<0.05$) among the different months with April month recording significantly lower value compared to January and August month. However, in the supplemented group, plasma insulin level was significantly lower ($P<0.05$) during summer month of April and May compared to winter months and rainy month of August. There was a significant increase ($P<0.05$) in the plasma insulin level in the supplemented group compared to the control group during all months of the study.

Table 22. Mean ± SE values of plasma cortisol (nmol/L) level in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	43.97 ± 1.32^{abA}	36.88 ± 1.65^{abA}	Winter	45.79 ± 1.09^{aA}	39.49 ± 1.23^{abB}
February	47.61 ± 1.31^{abA}	42.10 ± 1.83^{bcA}			
April	57.89 ± 3.28^{cA}	48.76 ± 1.14^{cB}	Summer	53.64 ± 1.98^{bA}	43.60 ± 1.34^{bB}
May	49.39 ± 3.08^{bA}	38.51 ± 2.01^{abB}			
July	43.09 ± 2.77^{abA}	36.77 ± 1.21^{abA}	Rainy	42.09 ± 2.37^{aA}	35.73 ± 0.70^{aB}
August	41.10 ± 2.43^{aA}	34.70 ± 1.31^{aA}			

The values with different superscripts within a column (a, b and c) and within a row (A and B) differ significantly ($P<0.05$).

Table 23. Mean ± SE values of plasma insulin (μIU/mL) level in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	22.97 ± 2.31^{bA}	34.38 ± 2.84^{bcB}	Winter	20.67 ± 1.23^{bA}	36.36 ± 1.47^{bB}
February	18.38 ± 1.14^{abA}	38.34 ± 2.07^{cB}			
April	14.82 ± 1.97^{aA}	27.05 ± 2.80^{aB}	Summer	15.65 ± 1.06^{aA}	26.00 ± 2.25^{aB}
May	16.49 ± 1.49^{abA}	24.95 ± 3.05^{aB}			
July	20.32 ± 1.32^{abA}	30.30 ± 1.23^{abB}	Rainy	20.89 ± 0.82^{bA}	32.29 ± 0.97^{bB}
August	21.46 ± 0.93^{bA}	34.28 ± 1.36^{bcB}			

The values with different superscripts within a column (a, b and c) and within a row (A and B) differ significantly ($P<0.05$).

Fig. 19. Plasma cortisol levels in Hallikar cattle during the study period.

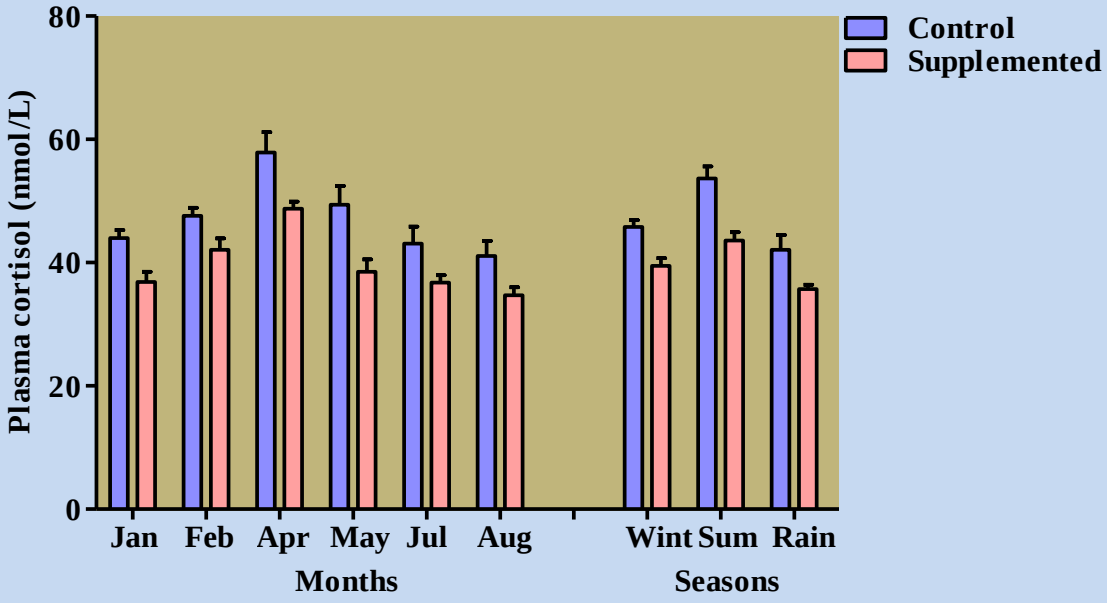
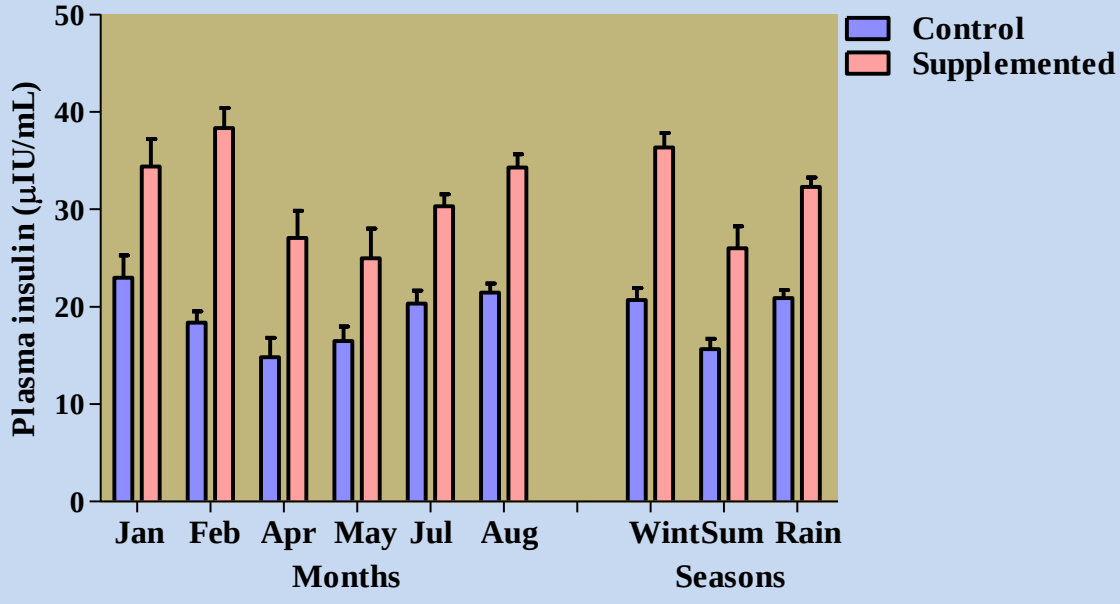


Fig. 20. Plasma insulin levels in Hallikar cattle during the study period.



Significantly lower (P<0.05) insulin level was recorded during summer season compared to winter and rainy season in both control and supplemented group. During all the seasons, insulin levels were significantly higher (P<0.05) in the supplemented group compared to control group.

4.4.6 Serum immunoglobulin (g/dL)

The values of serum immunoglobulin in Hallikar cattle during the study period are presented in Table 24 and Fig. 21.

The mean serum immunoglobulin level varied from 3.15 ± 0.15 to 4.67 ± 0.22 and 4.20 ± 0.34 to 5.68 ± 0.39 in control and supplemented groups, respectively. Significantly (P<0.05) higher level of serum immunoglobulin was recorded during winter months, summer months and rainy month of August compared July. The serum immunoglobulin level was significantly higher (P<0.05) in supplemented group compared to control group during all months of the study except during August.

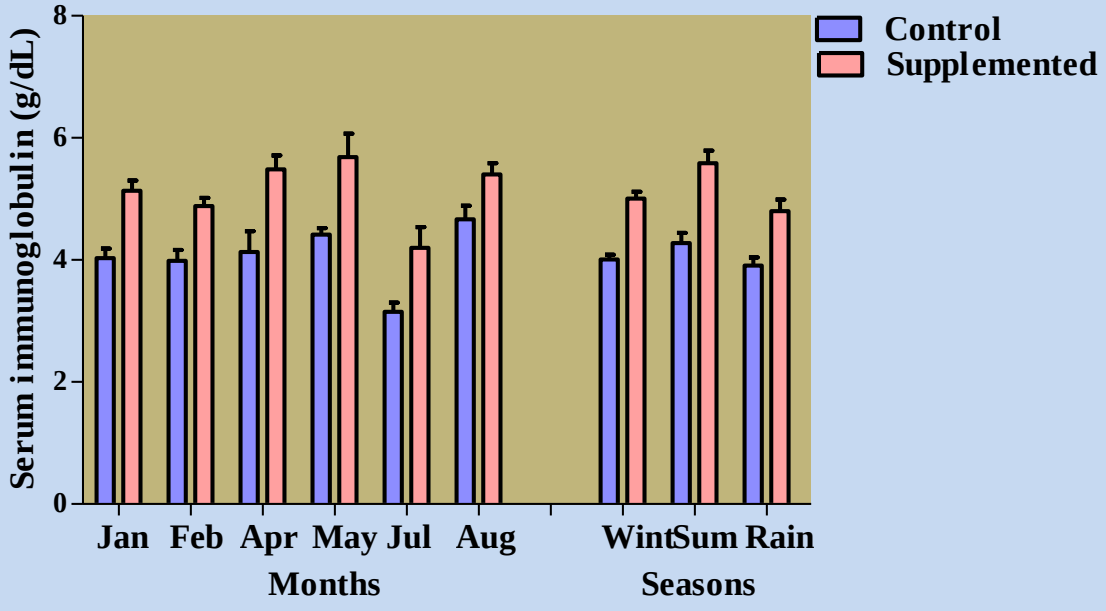
There was no significant (P>0.05) variation in the serum immunoglobulin level among the seasons in the control group, however, in supplemented group it was significantly higher (P<0.05) during summer season compared to other seasons. Further, the immunoglobulin levels were significantly (P<0.05) in supplemented group compared to control group during all the seasons.

Table 24. Mean ± SE values of serum immunoglobulin (Ig) level (g/dL) in Hallikar cattle during the study period (n = 6).

Months	Control Group	Supplemented Group	Seasons	Control Group	Supplemented Group
January	4.03 ± 0.15^{bA}	5.13 ± 0.17^{bcB}	Winter	4.01 ± 0.08^{aA}	5.01 ± 0.11^{aB}
February	3.98 ± 0.18^{bA}	4.88 ± 0.13^{abB}			
April	4.13 ± 0.34^{bA}	5.48 ± 0.23^{bcB}	Summer	4.28 ± 0.17^{aA}	5.58 ± 0.21^{bB}
May	4.42 ± 0.11^{bA}	5.68 ± 0.39^{cB}			
July	3.15 ± 0.15^{aA}	4.20 ± 0.34^{aB}	Rainy	3.91 ± 0.14^{aA}	4.80 ± 0.19^{aB}
August	4.67 ± 0.22^{bA}	5.40 ± 0.18^{bcA}			

The values with different superscripts within a column (a, b and c) and within a row (A and B) differ significantly ($P<0.05$).

Fig. 21. Serum immunoglobulin (Ig) levels in Hallikar cattle during the study period



Discussion

V. DISCUSSION

The effect of summer stress and dietary supplementation of vitamin E and selenium on the levels of HSP70, antioxidant enzyme activities, serum biochemical and hormonal profiles in Hallikar cattle are discussed in this chapter.

5.1 Temperature humidity index (THI)

The significant increase in THI observed during summer and rainy seasons compared to winter season during the course of the present study (Table 3) might be due to the higher ambient temperature and higher relative humidity, respectively. Al-Samawi *et al.* (2014), Trana *et al.* (2006) and Srikandakumar *et al*. (2003) also recorded significantly higher THI during summer season compared to the other seasons of the year. The THI value of 75 to 80 indicated moderate to high intensity of thermal stress (Cincovic *et al*., 2011) and THI of 72 and below was considered as no heat stress (cool), 73 to 77 as mild heat stress, 78 to 89 as moderate and above 90 as severe heat stress (Srikandakumar *et al*., 2003). Therefore, in the present study the THI of 69.97 during the winter season indicated that the animals were under no heat stress. However, the THI of 77.52 and 75.29 during the summer and rainy seasons indicated that the animals were under moderate to high intensity of thermal stress.

5.2 Plasma Heat shock Protein 70 (HSP70)

Significantly higher concentrations of plasma HSP70 recorded during the summer season compared to winter season in control group (Table 4 and Fig. 1) could be to provide better cellular protection against the deleterious effects of thermal stress and to

enhance the thermotolerance ability of the heat stressed animals as the heat tolerance at the cellular level is directly proportional to the ability of the cell to maintain the elevated levels of heat shock proteins, hallmark for the acclimation process, activation of heat shock responses in the cells leading to increased secretion of HSPs into the extracellular spaces and plasma (Roy and Collier, 2012). Significant increase in plasma HSP70 levels during summer season in control and supplemented group could also be attributed to increased levels of cortisol during the summer season in both the groups as cortisol is known to release the preformed HSP70 (bound to glucocorticoid receptors) when it binds to its receptors in the cytoplasm of the target cell (Behl *et al*., 2010). The higher levels of HSP70 recorded during summer in the present study might improve the cell survivability by reducing the accumulation of abnormal or damaged proteins in the cells and thus reducing the heat induced cellular apoptosis. Significantly higher HSP70 during summer recorded in the study was in accordance with the findings of Patir and Upadhyay (2007) and (2010) in buffaloes, Dangi *et al*. (2012) in goats, Gaughan *et al*. (2013) in cross bred cattle, Deb *et al*. (2014) in Sahiwal and Frieswal cattle and Kamwanja *et al*. (1994) in Angus, Brahman and Senepol breeds of cattle, who have recorded higher HSP70 levels during summer season.

Higher expression of HSP70 could stabilize the cells and maintain cellular homeostasis (Kamwanja *et al*., 1994; Patir and Upadhyay, 2010) and better thermotolerance ability of Sahiwal cattle was attributed to higher levels of HSP90 in summer (Prava and Upadhyay, 2014).

The nonsignificant variation in the plasma HSP70 levels during summer in both control and supplemented groups in the present study (Table 4 and Fig. 1) indicated that the vitamin E and selenium supplementation did not influence the plasma HSP70 levels in Hallikar cattle. This finding was in agreement with Zhang *et al*. (2014) who observed nonsignificant difference in HSP72 expression between the cows with or without chromium supplementation during high temperature humidity index period.

However, Sharma *et al*. (2013) showed significant up-regulation of HSP70 genes during heat stress in melatonin treated Barbari goats and Aggarwal *et al*. (2013) and Lallawmkimi *et al*. (2013) observed significant reduction in the plasma HSP70 levels in α-tocopherol acetate supplemented crossbreed cows and vitamin E supplemented Murrah buffaloes, respectively, during heat stress.

From the present study it was concluded that the enhanced expression of HSP70 during summer season and subsequent increase in its concentration in the plasma in Hallikar cattle could confer the better thermotolerance ability to maintain the cellular integrity during the thermal stress. Further, the supplementation of antioxidants did not influence the plasma HSP70 levels in heat stressed animals.

5.3 Antioxidant enzyme activities

5.3.1 Erythrocyte Catalase activity

Significantly higher ($P<0.05$) erythrocyte catalase activity during the summer season compared to winter season observed in the present study (Table 5 and Fig. 2) could be attributed to increased oxidative stress during summer season resulting in

generation of free radicals and subsequent stimulation of the endogenous antioxidant defense system to quench these reactive oxygen species. Catalase is heme-containing enzyme that catalyzes the dismutation of hydrogen peroxide into water and oxygen (Aggarwal and Prabhakaran, 2005) and increase in catalase and glutathione peroxidase activity is essential to alleviate the effects of H_2O_2 (Aggarwal *et al.,* 2013). The increased activity of catalase during summer could also be due to enhanced production of H_2O_2 as a result of increased activity of superoxide dismutase observed in the present study during summer. Similar findings were also reported by Kumar *et al*. (2007) in cattle and buffaloes, Kumar *et al*. (2011) in buffaloes, Ganaie *et al*. (2013a) in Murrah buffaloes, Lallawmkimi *et al*. (2013) in Murrah buffaloes and Yatoo *et al*. (2014) in buffaloes, who have reported significantly higher activity during summer season. However, Sunil Kumar *et al*. (2011) reported significant decrease in the erythrocyte catalase activity during heat stress in buffaloes and they attributed this decrease to the sparing of the endogenous antioxidant enzymes by the supplemented antioxidants.

Significant reduction in the erythrocyte catalase activity in the supplemented group during summer season observed in the present study indicated reduced oxidative stress in supplemented group compared to control group due to vitamin E and selenium mediated quenching of the free radicals generated during heat stress. Since vitamin E acts as a primary antioxidant of the cell membrane (Goff, 2005), its supply through diet could reduce reactive oxygen species and prevent the lipid peroxidation in the biological membrane thus resulting in reduced oxidative damage to the cells. Selenium being the constituent of many selenoproteins also help to reduce the toxic effects of reactive oxygen species (Sordillo, 2013). The significant reduction in the catalase activity in the

supplemented group was also reported by Kumar *et al*. (2011) and Lallawmkimi *et al*. (2013) who observed significantly lower catalase activity in buffaloes supplemented with ascorbate along with salt and vitamin E, respectively, compared to control group animals. Chandra and Aggarwal (2009) and Ganaie *et al.* (2013a) also observed significantly lower catalase activity in cows supplemented with DL-α-tocopherol and Murrah buffaloes supplemented vitamin C, respectively, compared to the control group animals.

5.3.2 Erythrocyte Superoxide dismutase (SOD) activity

In the present study, significant increase ($P<0.05$) in the erythrocyte SOD in the control group during the summer season (Table 6 and Fig. 3) could be due to heat stress induced generation of the free radicals especially superoxide anion and could also be due to auto-oxidation of hemoglobin resulting in generation of the superoxide anion in erythrocytes. Being considered as first line of defense against the prooxidants, the higher activity of erythrocyte SOD during summer might be aimed at preventing the oxidative injury to the cells. The result of the present study was in conformity with Trana *et al*. (2006) in dairy Red Syrian goats, Kumar *et al*. (2011) in buffaloes, Chigerwe *et al*. (2013) in neonatal dairy calves and Yatoo *et al*. (2014) in lactating and non-lactating cattle, who reported significantly higher SOD activity during summer. The activity of the antioxidant enzymes may increase or decrease as a consequence of enhanced ROS productions either by up-regulation of enzyme activity or utilization of the antioxidant enzymes to counter the ROS (Nazifi *et al*., 2009).

Superoxide dismutase is an important antioxidant enzyme in aerobic organisms that serve to scavenge the super oxide radical produced during oxidative stress resulting

in generation of H_2O_2 (Bernabucci *et al.* 2002). In conjugation with catalase and glutathione peroxidase, superoxide dismutase scavenges both intracellular and extracellular superoxide anion radicals and prevents lipid peroxidation (Aggarwal and Prabhakaran, 2005). However, Megahed *et al*. (2008), Sunil Kumar *et al*. (2011) and Sakatani *et al*. (2012) reported significant reduction in the SOD activity during summer in buffalo cows, buffaloes and Japanese Black cows, respectively.

Selenium can spare the vitamin E activity by scavenging the free radicals (Goff, 2004). Significant reduction in the erythrocyte superoxide dismutase activity in supplemented group could be attributed to sparing of the endogenous enzymatic antioxidants by the supplemented non enzymatic antioxidants during oxidative stress indicating the beneficial effects of antioxidants like vitamin E and selenium during the thermal stress. These findings are in agreement with the reports of Chandra and Aggarwal (2009) in cows, Sunil Kumar *et al*. (2011) in buffaloes, Kumar *et al*. (2011) in buffaloes, Ganaie *et al.* (2013a) in pregnant Murrah buffaloes and Lallawmkimi *et al.* (2013) in buffaloes, who reported significant decrease in the SOD activity in groups supplemented with DL-α-tocopherol, electrolytes along with ascorbic acid and zinc, ascorbate along with salts, vitamin C and vitamin E, respectively. However, Megahed *et al*. (2008) and Dimri *et al*. (2010) observed significant increase in the serum and erythrocyte SOD activity in antioxidant supplemented buffalo cows and water buffaloes, respectively.

5.3.3 Glutathione peroxidase (GPx) activity

GPx is a selenium dependent antioxidant enzyme that converts H_2O_2 to water (Ganaie *et al*., 2013b) and it also catalyzes the reduction of hydroperoxides formed from

fatty acids and other substances (Goff, 2005). Significantly higher ($P<0.05$) erythrocyte glutathione peroxidase activity in the control group during summer compared to other seasons observed in the present study (Table 7 and Fig. 4) could be due to stimulation of the body antioxidant system by increased levels of reactive oxygen species during summer. Higher GPx activity might also be due to increased generation of H_2O_2 by enhanced activity of SOD during summer as observed in the present study. The increased levels of glutathione peroxidase could be used as a sensitive marker of the oxidative stress in cattle. Similar observations were also made in the study of Bernabucci *et al*. (2002) in Holstein cows, Trana *et al*. (2006) in Red Syrian goats and Chigerwe *et al*. (2013) in neonatal dairy calves, who have recorded significantly higher GPx activity during summer compared to other seasons. Bernabucci *et al*. (2002) attributed this increase in GPx activity to the compensatory changes in cows in response to oxidative stress during the summer months. Chandra and Aggarwal (2009) opined that the dismutation of super oxide by enhanced SOD activity during the summer results in increased production of H_2O_2 and protection from this ROS would only be conferred by coordinated increase in the activity of catalase and glutathione peroxidase. However, Sakatani *et al*. (2012) reported a significant decrease in erythrocyte GPx activities in Japanese Black cows during summer season and was attributed to reduced lipid peroxidation resulting in reduced antioxidant response of erythrocytes.

The antioxidant function of vitamin E is synergistic with selenium. Selenium has been shown to act in aqueous cell media (cytosol and mitochondrial matrix) by destroying hydrogen peroxide and hydroperoxides via the enzyme glutathione peroxidase (McDowell, 2002). Further, a significant reduction in the erythrocyte GPx activity

observed in the supplemented group compared to the control group in the present study could be due to the fact that the vitamin E and selenium can scavenge the free radicals resulting in reduced oxidative stress in the supplemented group. This is in accordance with the findings of Ganaie *et al*. (2013a) who reported significant reduction in the plasma GPx activity in buffaloes supplemented with vitamin C.

But, the reports of Doni *et al.* (1984), Tanha *et al.* (2011), Calamari *et al*. (2011) and Deka *et al*. (2013) showed significant increase in the GPx activity in rats, Holstein cows, dairy cows and piglets when they were supplemented with selenium, glutamine, selenium with zinc and copper, respectively.

5.4 Serum biochemical profile

5.4.1 Serum aspartate aminotransferase (AST) activity

The activity of AST is high in the liver of all domestic animals and serum is used routinely in all species for the evaluation of the liver cell injury. AST activity is also high in kidney, heart and skeletal muscles (Kaneko *et al*., 1997). Significantly higher ($P<0.05$) serum AST activity during summer season in the control group observed in the present study (Table 8 and Fig. 5) could be due to free radicals induced damage to the hepatic tissue and possibly to the other tissues also leading to leakage of this intracellular enzyme into the blood stream resulting in its elevated level in the serum. As reported by Nazafi *et al*. (2003) in fat-tailed Iranian sheep, Srikandakumar and Johnson (2004) in Holstein, Jersey and Australian Milking Zebu cows, Rasooli *et al*. (2004) in Holstein heifers, Al-Saeed *et al*. (2009) in local cattle, Chandra Bhan *et al*. (2012) in Sahiwal cattle and Ashatsham-ul Haq *et al*. (2013) in dairy cows, higher AST activity during summer season

was observed compared to other seasons. Al-Saeed *et al*. (2009) and Chandra Bhan *et al*. (2012) attributed this increase in the AST activity to the oxidative stress induced cellular damage in the liver during summer months. However, Srikandakumar *et al*. (2003) reported a significant decrease in the plasma AST activity in both Merino and Omani sheep during heat stress and they ascribed this decrease to the slowdown of function of the liver enzymes due to reduced metabolism in animals exposed to heat stress.

Significant reduction in the serum AST activity in the supplemented group compared to the control group observed in the present study could indicate the hepatoprotective effect of the vitamin E and selenium that prevent the oxidative injury to the hepatic cells. Similar finding was also reported by Ashatsham-ul Haq *et al*. (2013) who reported a significant decline in the plasma AST activity in the summer stressed dairy cows supplemented with ascorbic acid and amla powder. However, study of Calamari *et al*. (2011) and Ganie *et al*. (2012) indicated that the supplementation of Italian Friesian lactating dairy cows and buffalo heifers with selenium and zinc, respectively, did not significantly influence the AST activity.

The results of the present study indicated that the significant increase in the serum AST activity during the summer in control group could be due to the hepatocellualr injury caused by free radicals induced lipid peroxidation and significant reduction in its activity in supplemented group could be due to vitamin E and selenium mediated protection of the cellular components from free radical induced lipid peroxidation.

5.4.2 Serum alanine aminotransferase (ALT) activity

Serum ALT could be used as one of the most universal markers for hepatic injury across species and its low concentration in peripheral circulation indicates normal cell turnover or release from nonvascular sources. But, their levels in the serum indicate hepatic injury (Amacher, 1998). In cattle higher concentration of this enzyme is found in liver and muscles (Kaneko *et al*., 1997). The significant ($P<0.05$) increase in the serum ALT activity observed during the summer season compared to other seasons (Table 9 and Fig. 6) could be attributed to heat stress induced oxidative damage to the liver cells that resulted in the leakage of this enzyme into the extracellular fluids. The higher levels of ALT during summer could also be due to muscle cell damage as some degree of muscle degradation also occurs during heat stress in cows (Wheelock *et al*., 2010). The findings of the present study were in agreement with the reports of Nazafi *et al*. (2003) in fat-tailed Iranian sheep, Al-Saeed *et al.* (2009) in white Fulani cows, Sharma and Kataria (2011) in Marwari goats, Alameen and Abdelatif (2012) in crossbred dairy cows, Chandra Bhan *et al*. (2012) and Pandey *et al*. (2013) in Sahiwal cattle, who have reported significant increase in ALT activity during summer season. However, Bahga *et al.* (2009) reported a significant decrease in serum ALT activity in crossbred calves during summer season compared to the spring season.

Significant reduction in serum ALT activity in supplemented group compared to the control group observed in the present study could be attributed to vitamin E and selenium mediated free radical scavenging and resultant reduction of free radical injury to liver and muscle tissue. This was in agreement with the study of Ashatsham-ul-Haq *et al*. (2013) who also recorded significant reduction in the ALT activity in ascorbic acid and

amla powder supplemented crossbred dairy cows. Further, Shashidhar and Prasad (1993) reported a significant increase in the serum ALT activity in goats supplemented with selenium and Devi *et al.* (2014) observed significantly higher serum ALT activity in zinc supplemented kids.

Therefore, the significant increase in the serum ALT activity observed in the present study could be an indication of oxidative injury to the hepatic cells during the summer months and the significant reduction in the ALT activity in the supplemented group indicated the beneficial hepatoprotective role of the antioxidants (vitamin E and selenium) supplied in the diet. The heat stress induced increase in the circulatory transaminase (AST and ALT) activity observed in the present study could also be due to the cortisol induced gluconeogenesis process as the transaminase play important role in amino acid catabolism.

5.4.3 Serum alkaline phosphatase (ALP) activity

Alkaline phosphatase activity is found in many tissues including bone, liver, intestine, kidney, placenta and germ cells (Sarkar, 2012). ALP is mainly localized in the cellular membrane of hepatocytes and increased levels of ALP in serum / plasma indicate liver intoxication or steroid hepatosis (Soch *et al*., 2008). ALP has been indicated to be a quick and reliable blood marker for heat stress in animals (Chandra Bhan *et al*., 2012)**.** Significantly higher ($P<0.05$) serum ALP activity in control group during summer season observed in the present study (Table 10 and Fig. 7) could be due to oxidative damage to the liver cell during the summer season and therefore ALP level in the blood could be used as an indicator of heat stress in cattle. Similar findings were reported by Rasooli *et*

al. (2004), Chandra Bhan *et al*. (2012) and Ashatsham-ul Haq *et al*. (2013) in HF heifers, Sahiwal cattle and crossbred dairy cows, respectively during summer season. Further, Nazafi *et al*. (2003), Srikandakumar *et al*. (2003), Abeni *et al.* (2007) and Abdel-Fattah (2014) reported significant decrease in ALP activity in Iranian sheep, Merino and Omani sheep, Holstein cows and Balady and Damascus goats, respectively. Significant decline in serum ALP activity was attributed to the slowdown of the liver enzyme function when the animals are exposed to heat stress (Srikandakumar *et al*., 2003) and to an increase in the plasma volume as a result of heat shock and subsequent increase in blood volume so as to maintain the homeothermy (Abdel-Fattah, 2014). Bahga *et al*. (2009) opined that the higher serum alkaline phosphatase activity during summer maintains homeostasis and generate energy in the animal body during heat stress.

The significant reduction ($P<0.05$) in the overall serum ALP activity in supplemented group compared to control group observed in the present study indicated the beneficial effect of antioxidants during the heat stress to minimize the hepatocellular damage is in accordance with Ashatsham-ul Haq *et al*. (2013) who showed significant reduction in the plasma ALP activity in crossbred cows supplemented with ascorbic acid and amla powder during summer season. Devi *et al.* (2014) showed a significant ($P<0.05$) increase in the serum activity of the alkaline phosphatase in zinc supplemented group of animals suggesting that the zinc as a micro-mineral stimulates the catalytic activity of the enzyme. Chandra Bhan *et al*. (2012) opined that the significant increase in plasma AST, ALT and ALP is mainly due to the leakage of theses enzymes from the liver cytosol into the blood stream, which reflects the liver damage and disruption of the normal liver function.

5.5 Serum protein profile

5.5.1 Serum total protein

Significantly lower serum total protein concentration observed during the summer season compared to winter season (Table 11 and Fig. 8) in control group could be due to impaired protein synthesizing capacity of the liver induced by oxidative damage to the hepatic cells. Reduction in the protein levels could also be due to higher levels of plasma cortisol observed in the present study as the catabolism of the proteins support the cortisol induced gluconeogenesis process. Protein synthesis in the hepatic cells depends on the concentration of circulating insulin in the body (Guyton and Hall, 2006) and reduced total protein levels during summer could also be attributed to the corresponding decline in the secretion of insulin during summer observed in the present study. The study of Nazafi *et al*. (2003) in sheep, Gudev *et al*. (2007a) in buffaloes, Omran *et al*. (2011) in Egyptian buffalo calves, Sharma and Puri (2013) in female Marwari goats and Sejian *et al.* (2013) in Malpura ewes also showed significant decline in the serum total protein level during summer season. However, Rasooli *et al*. (2004), Shrikhande *et al.* (2008) and Al-Haidary *et al*. (2012) have reported significantly higher levels of total protein during summer season in Holstein heifers, lactating cows and Najdi rams, respectively. The significant increase in the serum total protein could be due to the dehydration caused by enhanced respiratory rate during the summer months (Al-Haidary *et al*., 2012). But, Kumar *et al*. (2012) and Das *et al*. (2013) reported nonsignificant difference in serum total protein concentration in goats and Nili-Ravi buffaloes, respectively between the seasons indicating that the animals were unaffected by the heat stress.

The higher levels of serum total protein in the supplemented group compared to control group could be due to vitamin E and selenium mediated protection of the hepatic cells and maintenance of healthy liver. The increased levels could also be attributed to reduced cortisol levels and subsequent reduction in gluconeogenesis and sparing of the proteins. This finding was in agreement with results of Hala *et al*. (2009) who also observed significantly enhanced serum total protein concentration in buffaloes supplemented with only zinc and zinc plus Vitamin E / selenium. Abou-Zeina *et al*. (2009) also reported significantly higher levels of total protein in zinc, sodium selenite and vitamin E supplemented buffaloes. However, the supplementation of summer stressed Beetal goats with vitamin C and E-Se (Kumar *et al*., 2012) and Nili-Ravi buffaloes with selenium (Das *et al*., 2013) did not significantly influence the plasma total protein concentration.

5.5.2 Serum albumin

Non-significant variation ($P>0.05$) in the serum albumin levels between the seasons observed in the control group (Table 12 and Fig. 9) could be to enable the animal to maintain normal plasma osmotic pressure to maintain blood volume and body fluid distribution during heat stress. Das *et al*. (2013) also reported non significant difference in the albumin concentration between seasons in Nili-Ravi buffaloes.

However, Omran *et al*. (2011), Al-Haidary *et al*. (2012), Sejian *et al*. (2013) and Abdel-Fattah (2014) have reported significant reduction in albumin concentration during heat stress in Egyptian buffalo calves, Najdi rams, Malpura ewes and Balady and Damascus breeds of goats, respectively. As against the present study findings, Rasooli *et*

al. (2004) and Abdelatif *et al*. (2009) observed significant increase in serum albumin concentrations during summer compared to winter in Holstein heifers and in female Nubian goats, respectively. Shrikhande *et al.* (2008) also recorded increased serum albumin concentration in lactating cows and attributed this variation to various physiological, managemental and genetic factors. Non significant increase (P>0.05) in serum albumin in supplemented group compared control group observed in the present study was in agreement with Hala *et al*. (2009) who reported that the supplementation of the buffaloes with only zinc and zinc plus vitamin E / selenium did not influence the serum concentration of albumin significantly. However, Ganie *et al*. (2012) observed significant decline in serum albumin concentration in selenium supplemented group.

5.5.3 Serum globulin

The non-significant variation (P>0.05) in the serum globulin levels during different seasons in the control group (Table 13 and Fig. 10) observed in the present study is in agreement with the finding of Das *et al*. (2013) who also reported non-significant variation in plasma globulin concentration during different seasons in Nili-Ravi buffaloes. However, Abdelatif *et al*. (2009) in female Nubian goats and Al-Haidary *et al*. (2012) in Najdi rams have reported higher levels of globulins during hot summer compared to winter. Al-Haidary *et al*. (2012) attributed the increased globulin levels to the dehydration caused by enhanced breathing rate during the season of high temperature. Al-Saeed *et a*l. (2009), Sharma and Puri (2013) and Abdel-Fattah (2014) found significant decrement in serum globulin levels in heat stressed local Iranian cattle, female Marwari goats and Balady and Damascus breeds of goats, respectively.

Significant increase (P<0.05) in the globulin levels in supplemented group compared to control group during summer season was similar to the findings of Ganie *et al*. (2012) who observed significant increase in serum globulin levels in selenium supplemented buffalo heifers. Abou-Zeina *et al*. (2009) also reported significantly higher levels of serum globulins in zinc, sodium selenite and vitamin E supplemented buffaloes.

5.6 Serum lipid profile

5.6.1 Serum triglycerides

Serum triglycerides level was significantly lower (P<0.05) in summer season compared to winter season in the control group (Table 14 and Fig. 11). This reduction in serum triglycerides could be attributed to the increased hormone sensitive TG-lipase as a result of enhanced secretion of the cortisol during summer season in control group in the present study. Also, insulin inactivates the hormone sensitive TG-lipase by decreasing the cAMP production (Satyanarayana and Chakrapani, 2007) and the lower triglycerides during summer could be due to higher lipase activity due to reduced insulin secretion during summer season observed in the present study. Omran *et al*. (2011) and Pandey *et al*. (2012) also reported significant decline in the plasma and serum triglycerides concentration during heat stress in Egyptian buffalo calves and Marwari goats, respectively.

Significant increase (P<0.05) in serum triglyceride concentration in supplemented group compared to control group could be the result of reduced lipolysis effected by lowered cortisol levels and higher insulin levels in the supplemented group observed in the present study. Kumar *et al*. (2007) observed non significant variation in plasma

triglyceride concentration between the control and vitamin C and E-Se supplemented Beetal goats during summer stress.

5.6.2 Serum total cholesterol

Cholesterol acts as a precursor for the synthesis of steroid hormones in the body and the significantly higher levels of circulating cholesterol in control group during summer season (Table 15 and Fig. 12) compared to winter season could be due to the increased body demand for the cholesterol for increased cortisol synthesis and secretion that occurs during summer stress. The finding was in agreement with the reports of Sinha *et al*. (1981) in Jersey and Red Dane cattle, Kumar *et al*. (2012) in Beetal goats, Sejian *et al*. (2013) in Malpura ewes and Das *et al*. (2013) in buffaloes. Further, the significant increase in circulating plasma cholesterol could be needed to support the hepatic gluconeogenesis to supply the glucose for the adaptive mechanisms (Sejian *et al*., 2013). Higher cholesterol levels during high environmental temperature could be due to depressed activity of the thyroid gland during summer season (Sinha *et al*., 1981). The lipolytic glucocorticoids stimulate the fat mobilization from adipose tissue and increase the circulating concentrations of the free fatty acids. Thus, an increase in the concentration of the serum cholesterol would be expected after a stress episode (Abdel-Fattah, 2014). However, Rasooli *et al*. (2004), Gudev *et al*. (2007a), Cincovic *et al*. (2011) and Pandey *et al*. (2012) reported significant decline in the cholesterol during the summer season in Holstein heifers, in Bulgarian buffaloes, Egyptian buffalo calves and Marwari goats, respectively. Lowered cholesterol level during the heat stress was attributed to lowered thyroid activity (Pandey *et al*., 2012), decreased feed intake during

hot summer and consequent reduction in intake of dietary cholesterol (Gudev *et al*., 2007a).

Supplemented group showed significantly higher ($P<0.05$) cholesterol levels compared to control group during all seasons, which could be attributed to the lipotropic effects of vitamin E supplemented through the diet during entire period of the study. This is in conformity with the observations of Zhang *et al*. (2014) who found significantly higher concentration of serum cholesterol in chromium supplemented Holstein cows during hotter period of the year.

5.6.3 Serum HDL cholesterol, LDL cholesterol and VLDL cholesterol

HDL-cholesterol, a lipoprotein complex concerned with the reverse transportation of cholesterol, traps cholesterol of peripheral tissues and transport it to the liver for degradation and elimination from the body and hence it is considered as good cholesterol (Satyanarayana and Chakrapani, 2006). Significantly higher HDL-cholesterol levels (Table 16 and Fig. 13) in the supplemented group compared to control group during all the seasons observed in the present study indicated the beneficial effect of antioxidant supplementation. The higher HDL-cholesterol in supplemented group could be attributed to significantly higher levels of total cholesterol in that group compared to control group. The higher HDL-cholesterol in supplemented group could also be attributed to the correspondingly higher levels of LDL-cholesterol in that group as the HDL can function as LDL antioxidant (Tomas *et al*., 2004). This was in agreement with Das *et al*. (2013) who reported significantly higher plasma HDL-cholesterol levels in buffaloes

supplemented with niacin, yeast and mustard oil and they have attributed the same to the combined effects of supplementation and managemental factors.

The plasma cholesterol is associated with the different lipoprotein fractions such as LDL, VLDL and HDL cholesterol (Satyanarayana and Chakrapani, 2006). Significantly higher ($P<0.05$) levels of LDL-cholesterol recorded during summer and rainy seasons (Table 17 and Fig. 14) in control group could be due to corresponding higher levels of circulating total cholesterol observed in the present study during summer and rainy season. As the transportation of vitamin E demands higher levels of lipoproteins (VLDL and LDL) in the body (Satyanarayana and Chakrapani, 2006), significantly higher LDL cholesterol in supplemented group compared to control group could be due to higher levels of vitamin E in that group

VLDL cholesterol is involved in transportation of triacylglycerol from the liver to the adipose tissue (Satyanarayana and Chakrapani, 2006). Therefore, the lower levels of VLDL cholesterol observed during summer in control group (Table 18 and Fig. 15) could be attributed to the corresponding lower levels of triglycerides in the control group observed in the present study. The significant increase in the serum VLDL cholesterol in the supplemented group compared to control group could be attributed to higher levels of triglycerides in that group.

5.7 Blood glucose

Significantly elevated blood glucose levels observed in the control group during summer season in the present study (Table 19 and Fig. 16) could be due to reduced utilization of glucose by the cells owing to the reduced insulin concentration (15.65 ±

1.06 μIU/mL) which was also observed in the present study during summer season, as the heat stressed animals maintain insulin-dependent glucose utilization compared to animals of thermoneutral region (Behl *et al*., 2010). During the periods of heat stress, hepatic glucose output increases as a result of increased glycogenolysis and increased gluconeogenesis (Rhoads *et al*., 2013a). Gluconeogenesis also occurs in mammalian kidney and heat stress induced release of ketone bodies accelerates the renal gluconeogenesis during summer. Although ketone bodies are weakly gluconeogenic, they act as fuel for respiration to spare the glucogenic substrates for gluconeogenesis (Salama *et al*., 2013). The present study finding was in agreement with the findings of Srikandakumar *et al*. (2003) in Merino sheep, Srikandakumar and Johnson (2004) in Holstein and Australian Milking Zebu cows, Trana *et al*. (2006) in Red Syrian goats, Avendano-Reyes *et al*. (2010) in Holstein cows, Al-Haidary *et al*. (2012) in Najdi rams, Ashatsham-ul Haq *et al*. (2013) in crossbred dairy cows and Sejian *et al*. (2013) in Malpura ewes.

However, study of Habeeb *et al*. (1996) in Friesian cows, Abeni *et al.* (2007) and Rasooli *et al*. (2004) in Holstein cows, Al-Saeed *et al*. (2009) in local cows of Iraq, Bahga *et al.* (2009) in crossbred calves, Cincovic *et al*. (2011) in Holstein cows, Pandey *et al*. (2012) in Marwari goats and Kumar *et al*. (2012) in female Beetal goats have observed significant reduction in the glucose levels during summer season compared to other seasons.

It was concluded that the significant increase in the blood glucose level during summer season could be due to stress induced increase in cortisol secretion and

consequent stimulation of gluconeogenesis, stress induced enhancement in glycogenolysis, renal gluconeogenesis and also could be due to inhibition of cellular glucose uptake and utilization during summer.

In the present study, it was also observed that the supplemented group showed significantly lower ($P<0.05$) glucose levels compared to control group during all three seasons. This could be the result of reduced heat stress in the vitamin E and selenium supplemented animals leading to enhanced release of insulin and subsequent enhancement of utilization of the glucose by the body cells. This decline in the glucose level might also be attributed to the insulin independent utilization of glucose by animals when heat stress is alleviated by the antioxidant supplementation. This was in accordance with Ashatsham-ul Haq *et al*. (2013) who have shown significant reduction in the plasma glucose levels in crossbred animals supplemented with ascorbic acid and amla powder and they attributed the decline in plasma glucose levels to ascorbic acid induced increase in insulin concentration and decrease in cortisol concentration. But, Kumar *et al*. (2012) showed significant increase in the plasma glucose concentration in vitamin C and vitamin C + E-Se supplemented goats.

5.8 Plasma hormone profile

5.8.1 Plasma triiodothyronine

The significant ($P<0.05$) reduction in plasma triiodothyronine level during summer season compared to winter season in control group (Table 20 and Fig. 17) might be due to lowered body metabolic rate during summer months which is required to maintain equilibrium between heat production and heat dissipation during summer stress.

This reduction could also be attributed to the negative effect of summer ambient temperature on the thyroid function and thyroid hormone levels (Todini, 2007). The reduced thyroid hormone levels during summer could also help the animals to acclimatize to heat stress as the reduced thyroid concentration reduces the consumption by cell and reduced heat production by the animal (Shido and Sakurada, 1993). Physiological response to heat stress involves reduced heat production which is largely achieved by reduced feed intake and thyroid hormone secretion (Hansen, 2004). Similar findings were reported by Magdub *et al*. (1982) in lactating cows, Collier *et al*. (1982) in heat stressed cows, Rasooli *et al.* (2004) in Holstein heifer, Sivakumar *et al*. (2010) in heat stressed goats, Omran *et al*. (2011) in buffalo calves, Banerjee *et al*. (2013a) in Gaddi, Chegu, Sirohi and Barbari breeds of goats, Sejian *et al.* (2013) in Malpura ewes and Al-Samawi *et al*. (2014) in female Aardi goat. However, Habeeb *et al*. (1996) and Alameen and Abdelatif (2012) reported significantly higher serum concentrations of triiodothyronine during summer season in crossbred dairy cows and Iranian flat-tailed sheep, respectively. Das *et al*. (2014) found no significant differences in T_3 levels between control and treated groups.

Though the plasma T_3 levels in the supplemented group were significantly lower compared to control group, they were well within the normal range (0.64 to 1.0 ng/mL) reported for animal species of veterinary importance (Eiler, 2005). This indicated that the animals in the supplemented group were not suffering from the heat stress and thus could maintain the normal metabolic rate with normal circulating T_3 levels. But, the study of Hala *et al*. (2009) in buffaloes and Sivakumar *et al*. (2010) in goats revealed a significant increase in the serum concentrations of T_3 in supplemented group.

Higher levels of T_3 recorded in control group could be due to increased conversion of T_4 to T_3 which was evidenced by the corresponding lower T_4 levels in the control group when compared to the reference values of T_4 (15-40 ng/mL) for animals (Eiler, 2005).

5.8.2 Plasma thyroxine

Significantly lower ($P<0.05$) concentrations of plasma thyroxine observed in control group during summer season compared to winter and rainy seasons (Table 21 and Fig. 18) in the present study was in agreement with the reports of Magdub *et al*. (1982), Collier *et al*. (1982), Habeeb *et al*. (1996), Rasooli *et al.* (2004), Alameen and Abdelatif. (2012), Banerjee *et al*. (2013a) and Al-Samawi *et al*. (2014). The reduced thyroxine level during summer could be attributed to reduced T_4 synthesizing capacity of the thyroid follicular cells during summer. Declined T_4 synthesizing capacity in turn could be attributed to the reduced availability of thyro-peroxidase enzyme (required for oxidation of iodide ions to form iodine atoms) as much of the enzyme activity is involved in catalyzing H_2O_2 generated summer stress (Sivakumar *et al*. (2010).

Significantly higher ($P<0.05$) plasma thyroxine concentration in supplemented group compared to control group during summer season in the present study indicated that vitamin E and selenium supplementation had alleviated the heat stress to enable the animal to adapt to the condition even at higher levels of thyroxine. This is in agreement with Hala *et al*. (2009) who observed a significant increase in the serum concentrations of T_4 in buffaloes fed with zinc methionine and vitamin E/Se + Zn methionine. Sivakumar

et al. (2010) also reported significant increase in plasma levels of thyroxine in goats supplemented with vitamin E + selenium and vitamin C.

5.8.3 Plasma cortisol

Significantly higher (P<0.05) concentration of plasma cortisol recorded during the summer season compared to other seasons (Table 22 and Fig. 19) in the control group indicated a state of thermal stress. The increased cortisol levels could be due to the activation of hypothalamic-pituitary-adrenal axis and consequent increase in plasma glucocorticoids including cortisol (Marai *et al*., 2007). Glucocorticoids released during thermal stress are associated with modifying the intracellular heat shock response. Binding of glucocorticoids to their cytoplasmic receptors could result in release of preformed cytoplasmic HSP70 and HSP90 which in turn provide an instant pool of HSP to prevent the protein denaturation due to oxidative stress (Behl *et al*., 2010). The findings of the study were in agreement with the observations made by Megahed *et al*. (2008) in buffalo cows, Sunil Kumar *et al. (*2010) in buffaloes, Sivakumar *et al.* (2010) in Black Bengal goats, Soltan (2010) in lactating dairy cows, Chandra Bhan *et al*. (2012) in growing and adult Sahiwal cattle, Sharma *et al.* (2013) in goats, Sejian *et al.* (2013) in Malpura ewes and Al-Samawi *et al*. (2014) in Ardi-goats.

There was significant reduction (P<0.05) in the plasma cortisol concentration in the supplemented group compared to the control group during summer months which could be due to reduced thermal stress owing to the antioxidant benefits of vitamin E and selenium. Similar observations were reported by Sivakuamr *et al*. (2010) in vitamin C, vitamin E and selenium supplemented goats, Soltan (2010) in chromium supplemented

lactating dairy cows and Sharma *et al.* (2013) in goats treated with melatonin. They have ascribed the decrease of cortisol levels to the amelioration of heat stress upon antioxidant supplementation.

5.8.4 Plasma insulin

In present study, the plasma insulin concentration was significantly lower ($P<0.05$) in control group during summer season compared to other seasons (Table 23 and Fig. 20). This could be attributed to reduced hormone synthesis due to reduced food intake during summer. This was in agreement with the findings of Johnson *et al*. (1988), Itoh *et al*. (1998) and Omran *et al*. (2011) who showed significant reduction in plasma insulin level in lactating cows and Egyptian buffaloes, respectively, during summer season compared to other seasons.

There was significant increase ($P<0.05$) in the plasma insulin concentration in the vitamin E and selenium supplemented group compared to control group during different seasons of the study and this could be attributed to reduction of the heat stress in antioxidant supplemented group that caused the enhanced feed intake and improved levels of insulin in such animals.

5.9 Serum immunoglobulins

Non signifcant variation ($P>0.05$) in the serum immunoglobulins level in the control group during different seasons indicated that the season did not exert any influence on the level of immunoglobulins. Higher levels of immunoglobulins in (Table 24 and Fig. 21) supplemented group compared to control group could be attributed to

vitamin E mediated protection of immune cells from lipid peroxidation resulting in increased immunoglobulin production in the supplemented group. This is in agreement with significantly higher serum immunoglobulin levels observed in buffaloes supplemented with zinc in combination with vitamin E or selenium (Hala *et al.,* 2009) and crossbred cows supplemented with alpha-tocopherol acetate (Aggarwal *et al.*, 2013).

Summary

VI. SUMMARY

The present study was conducted to ascertain the effect of summer stress and effect of supplementation of antioxidants such as vitamin E and selenium on plasma levels of HSP70, erythrocyte antioxidant enzymes activity, serum biochemical profile and plasma hormonal profile in Hallikar cattle. The study was conducted at Madabal Village of Magadi Taluk, Ramanagara District. A group of twelve recently calved female Hallikar cattle aged between 4 to 6 years were randomly selected and divided into control group and supplemented group, with six animals in each group. Animals of both the groups were exposed to natural environmental stressors for three different periods of the year such as winter months (January and February, 2014), summer months (April and May, 2014) and rainy months (July and August, 2014) by allowing them for grazing in agricultural fields during the day hours (7 hours/day). Animals of control group did not receive any supplementation. Supplemented group received vitamin E (1.47 g/animal/day) and selenium (0.007 g/animal/day) for the entire period of the study in addition to the regular maintenance diet. Blood samples were collected at monthly intervals during the study period from each animal of both the groups. Serum, plasma and erythrocyte suspension obtained from the collected blood samples were utilized for the determination of various parameters.

Plasma HSP70 levels were significantly higher during summer in both the control and supplemented groups and this elevated level of HSP70 could be attributed to the higher plasma cortisol levels during summer season compared to other seasons in both the groups. This enhanced HSP70 levels could confer better thermotolerance ability as it

always corresponds to capacity of the cells to maintain higher levels of HSPs. The non significant variation in the plasma HSP70 levels in supplemented group compared to control group indicated that the supplementation with vitamin E and selenium did not influence the plasma HSP levels.

There was a significant increase in the activities of antioxidant enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) in erythrocytes during the summer season compared to the winter and rainy seasons in the control group. The activities of these enzymes were significantly reduced during summer season in animals supplemented with vitamin E and selenium compared to control group animals. The results of the study indicated that the increase in the activities of antioxidant enzymes could be due to increased oxidative stress during summer. The significant increase in the SOD activity could scavenge the superoxide radicals generated as a result of increased oxidative stress during summer months. As the higher SOD activity increases the production of H_2O_2, the protection from generated ROS could be provided by the coordinated increase in the activity of catalase and superoxide dismutase. The reduction in the activity of these antioxidant enzymes in supplemented group compared to control group could be due to reduced oxidative stress by the supplemented antioxidants.

Serum activities of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were significantly increased during the summer season compared to winter and rainy season in control group animals. Vitamin E and selenium supplementation significantly reduced the serum transaminases and ALP activities in supplemented group compared to control group during summer season. The present study

indicated that the oxidative stress induced hepatocellular and muscle injury during summer season in control group resulted in increased activity of these enzymes and alleviation of the oxidative stress due to supplementation of antioxidants leading to significant reduction of these enzyme activities in the supplemented group.

Compared to control group, in the supplemented group, there was a significant increase in serum total protein and serum globulin levels during the summer season. But, serum albumin levels did not vary significantly between control and supplemented group during summer season. The significant reduction in the serum total protein in the control group during summer could be due to impaired protein synthesizing capacity, reduced feed intake, reduced insulin (anabolic hormone) secretion and increased cortisol (catabolic hormone) secretion during summer. In supplemented group, improved protein synthesizing capacity of liver due to reduced oxidative stress, improved feed intake, reduced secretion of glucocorticoids and enhanced secretion of insulin might have increased the levels of serum total protein.

Significantly lower levels of triglycerides during summer in the control group observed in the present study could be attributed to increased lipolytic activity owing to higher cortisol levels and lower insulin levels. Significant increase in triglycerides during summer in supplemented group compared to control group was attributed to reduced lipolysis due to reduced cortisol levels and increased insulin release. Significantly higher levels of serum cholesterol during summer compared to winter in the control group could be due to increased demand for cholesterol as a precursor for the enhanced cortisol synthesis that occurs in response to the summer stress. Significant increase in the

cholesterol levels in supplemented group compared to control group could be attributed to the lipotropic nature of the vitamin E supplemented in the diet. Higher HDL cholesterol in control group could be attributed to the higher levels of total cholesterol during summer. Further, the increase in HDL cholesterol in supplemented group could be attributed to increased levels of total cholesterol and LDL cholesterol levels in that group observed in the present study. Reduced VLDL cholesterol during summer in control group could be ascribed to lower level of triglycerides. The significant increase in VLDL cholesterol in supplemented group during summer could be attributed to higher triglyceride levels.

The enhanced blood glucose levels during summer could be attributed to enhanced hepatic glycogenolysis, hepatic gluconeogenesis, renal gluconeogenesis and reduced insulin-dependent utilization of glucose. Significant decline in the blood glucose levels in supplemented group compared to control group could be due to enhanced utilization of glucose owing to increased insulin release and reduced gluconeogenesis that finally resulted in reduced cortisol levels in the supplemented group.

Significant reduction in plasma levels of triiodothyronine and thyroxine during summer season could be beneficial to the animals to adjust their body temperature by way of reduced body metabolism in control group. Reduced levels of thyroxine and triiodothyronine in antioxidants supplemented group compared to control group indicated reduced oxidative stress during summer. Significantly higher T_3 levels in the control group indicated enhanced conversion of T_4 to T_3 as evidenced by corresponding decline in the T_4 below the normal range in control group.

Significantly higher levels of plasma cortisol during summer season in control group animals could be attributed to activation of hypothalamic-pituitary-adrenal axis which is considered as the most important response in animals exposed to various climatic stressors. Significantly higher levels of cortisol during summer could also help to release of preformed cytoplasmic HSP70 to enhance thermotolerance ability of the animals during the period of heat stress.

There was a significant decline in plasma insulin concentration during summer in control group which could be attributed to reduced feed intake in the stressed animals. However, significant increase of insulin levels in supplemented group compared to control group might encourage the utilization of glucose as energy source which was evident by the corresponding reduction in the blood glucose concentration in the supplemented group compared to control group in the present study.

Significant increase in serum immunoglobulin levels in supplemented group compared to control group indicated the antioxidants mediated immunopotentiation during summer.

CONCLUSIONS

1. Summer stress has induced higher levels of plasma HSP70 that conferred better thermotolerance ability in heat stressed animals and antioxidant supplementation did not influence the plasma HSP70 levels.
2. Higher activities of antioxidant enzymes indicated more oxidative stress during summer in control animals and the reduced antioxidant enzyme activities in supplemented group indicated the alleviation of oxidative stress by vitamin E and selenium.
3. Higher levels of serum enzymes such as AST, ALT and ALP indicated the oxidative stress induced hepatocellular injury in control group and their reduced levels in supplemented group indicated antioxidants mediated protection of the liver tissue.
4. Lowered serum protein levels during summer season indicated impaired protein synthesis in control group and significant increase in the protein levels in the supplemented group indicated improved protein synthesis in antioxidants supplied animals.
5. Reduced triglycerides levels during summer indicated enhanced utilization of triglycerides over glucose and significant increase in their levels in the supplemented group indicated lipotropic effects of vitamin E and selenium that added the lipids of the tissues into circulation.
6. Decreased thyroid hormone levels during summer compared to winter could help in the reduction of body metabolic rate and heat load on the animals and thus

enable the animal to cope up with higher ambient temperature. Normal levels of thyroid hormones in the supplemented group indicated alleviation of the heat stress.

7. Significant elevation in the plasma cortisol levels indicated oxidative stress during summer in control group and the reduced levels in supplemented group indicated reduced stress.
8. Heat stress reduced the insulin levels and thus reducing the utilization of glucose in the affected animals but antioxidants improved the insulin secretion levels during summer thus improving the insulin dependent glucose utilization.
9. Enhanced immunoglobulin levels in supplemented group compared to control group during summer season indicated the better immunity in supplemented animals.

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Abstract

VIII. ABSTRACT

The study was conducted to ascertain the effect of summer stress on heat shock protein 70 (HSP70) levels, erythrocyte antioxidant enzyme activities, serum biochemical and plasma hormonal profiles in Hallikar cattle. Twelve female Hallikar cattle aged four to six years belonging to farmers of Madabal village, Magadi Taluk, Ramanagara District, exposed to natural stressors were selected for the study and were divided into control and supplemented groups with six animals in each group. Control group received regular diet and supplemented group received vitamin E and selenium, additionally. Blood samples collected at monthly intervals during winter, summer and rainy seasons were utilized for determination of various parameters. Plasma HSP70 levels increased significantly in control and supplemented groups during summer seasons. Activities of catalase, superoxide dismutase, glutathione peroxidase, alanine transaminase, aspartate transaminase and alkaline phosphatase were significantly higher during summer season in control group that reflected summer stress compared to supplemented group. Serum total protein and triglycerides levels were significantly lower during summer in control group and increased significantly in supplemented group. The levels of triiodothyronine, thyroxine and insulin were significantly lower during summer in control group and increased significantly in supplemented group. Plasma cortisol level was significantly higher in control group and decreased in supplemented group. It was concluded that significantly higher plasma HSP70 levels during summer season confer better thermotolerance ability of the heat stressed animals. The reduced antioxidant enzyme activity and plasma cortisol levels in the supplemented group indicated the alleviation of heat stress by vitamin E and selenium supplementation.

Key words: HSP70, Antioxidant enzymes, Cortisol, Summer stress, Thermotolerance.

Appendices

IX. APPENDICES

Appendix 1. Radioimmunoassay flow chart for T_3

Tube No.		Buffer (µl)	Free serum (µl)	Standard / Sample (µl)	^{125} I – T_3 (µl)	Antiserum Complex (µl)
T_1	T_2 (Total)	-	-	-	100	-
1	2 (Blank)	350	50	-	100	-
3	4 (Zero)	250	50	-	100	100
5	6	200	50	50	100	100
7	8	200	50	50	100	100
9	10	200	50	50	100	100
11	12	200	50	50	100	100
13	14	20	50	50	100	100
15	16	250	-	50	100	100
17	18	250	-	50	100	100

Appendix 2. Radioimmunoassay flow chart for T_4

Tube No.		Buffer (µl)	Free serum (µl)	Standard / Sample (1:10) (µl)	^{125} I – T_4 (µl)	Antiserum (µl)
T_1	T_2 (Total)	-	-	-	100	-
1	2 (Blank)	200	100	-	100	-
3	4 (Zero)	100	100	-	100	100
5	6	-	100	100 (D)	100	100
7	8	-	100	100 (C)	100	100
9	10	-	100	100 (B)	100	100
11	12	-	100	100 (A)	100	100
13	14	100	-	100 (CA)	100	100
15	16	100	-	100 (CB)	100	100
17	18	100	-	100 (S)	100	100

Appendix 3. Radioimmunoassay flow chart of Insulin

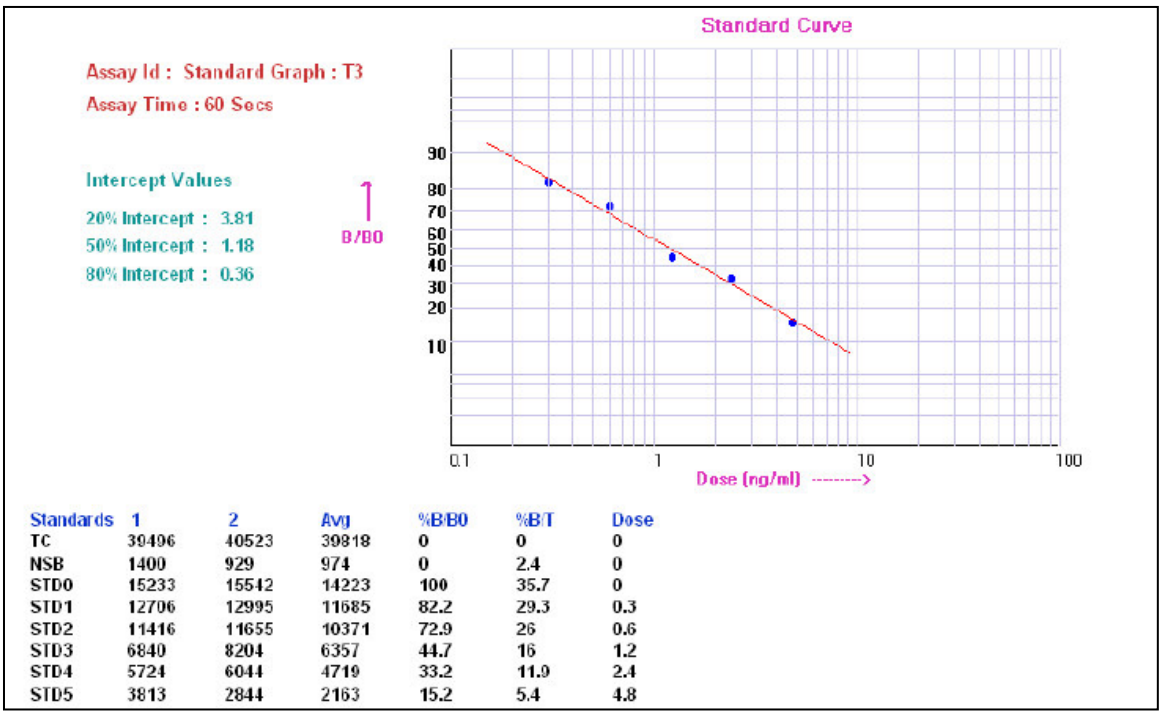
Tube No.		Buffer (mL)	Std. / Sample (mL)	Free serum (mL)	Anti serum (mL)	All the tubes mixed gently and incubated overnight at 4 °C	^{125} I – Insulin	All the tubes mixed gently and incubated at room temperature for 3 h	PEG (mL)
1	2 (Total)	-	-	-	-		0.1		-
3	4 (Blank)	0.4	-	0.1	-		0.1		1
5	6 (Zero)	0.3	-	0.1	0.1		0.1		1
7	8 (Std F)	0.2	0.1 (**F)**	0.1	0.1		0.1		1
9	10 (Std E)	0.2	0.1 (**E)**	0.1	0.1		0.1		1
11	12 (Std D)	0.2	0.1 (**D)**	0.1	0.1		0.1		1
13	14 (Std C)	0.2	0.1 (**C)**	0.1	0.1		0.1		1
15	16 (Std B)	0.2	0.1 (**B)**	0.1	0.1		0.1		1
17	18 (Std A)	0.2	0.1 (**A)**	0.1	0.1		0.1		1
19	20 (CA)	0.3	0.1(**CA)**	-	0.1		0.1		1
21	22 (CB)	0.3	0.1(**CB)**	-	0.1		0.1		1
21	22 (S1)	0.3	0.1 (**S1)**	-	0.1		0.1		1

(Note: Std. = Standard)

Appendix 4. Radioimmunoassay flow chart of Cortisol

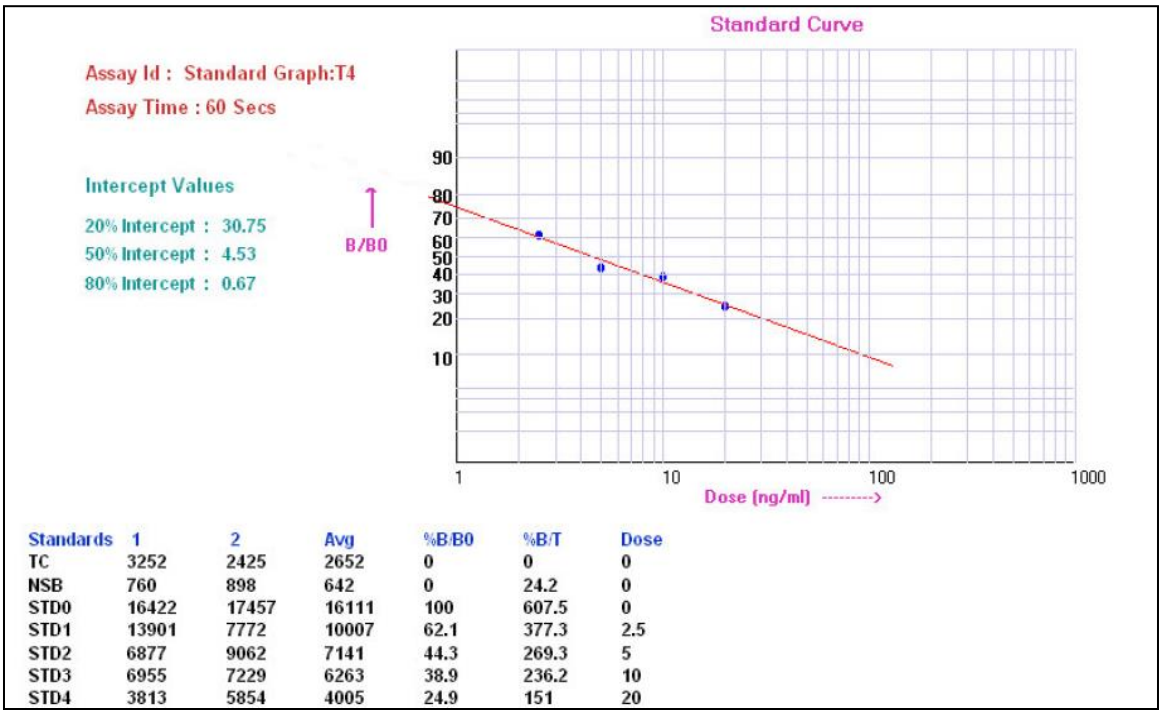
Tube No.		Calibrator / Control (µl)	^{125} I – Cortisol (µl)
T_1	T_2 (Total)	-	500
1	2 (Zero)	-	500
3	4	50 (E)	500
5	6	50 (D)	500
7	8	50 (C)	500
9	10	50 (B)	500
11	12	50 (A)	500
13	14	50 (Control)	500
15	16	50 (S_1)	500
17	18	50 (S_2)	500

Appendix 5. Standard curve for radioimmunoassay of plasma T_3



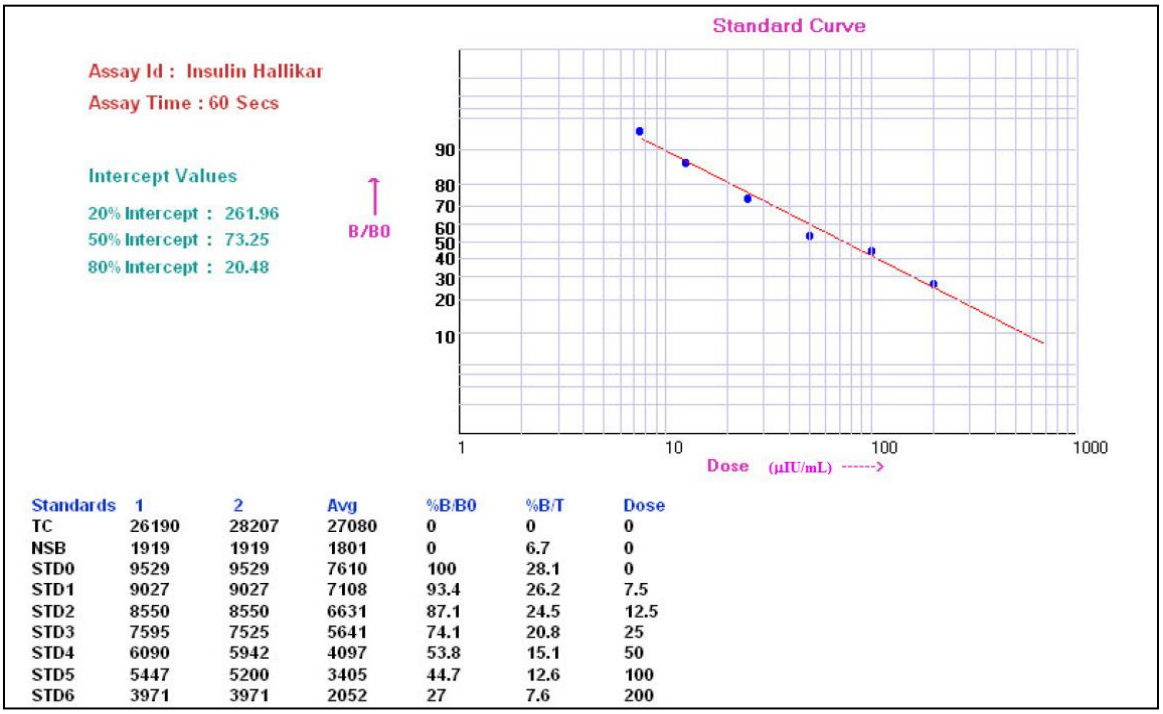
Standards	1	2	Avg	%B/B0	%B/T	Dose
TC	39496	40523	39818	0	0	0
NSB	1400	929	974	0	2.4	0
STD0	15233	15542	14223	100	35.7	0
STD1	12706	12995	11685	82.2	29.3	0.3
STD2	11416	11655	10371	72.9	26	0.6
STD3	6840	8204	6357	44.7	16	1.2
STD4	5724	6044	4719	33.2	11.9	2.4
STD5	3813	2844	2163	15.2	5.4	4.8

Appendix 6: Standard curve for radioimmunoassay of plasma T_4



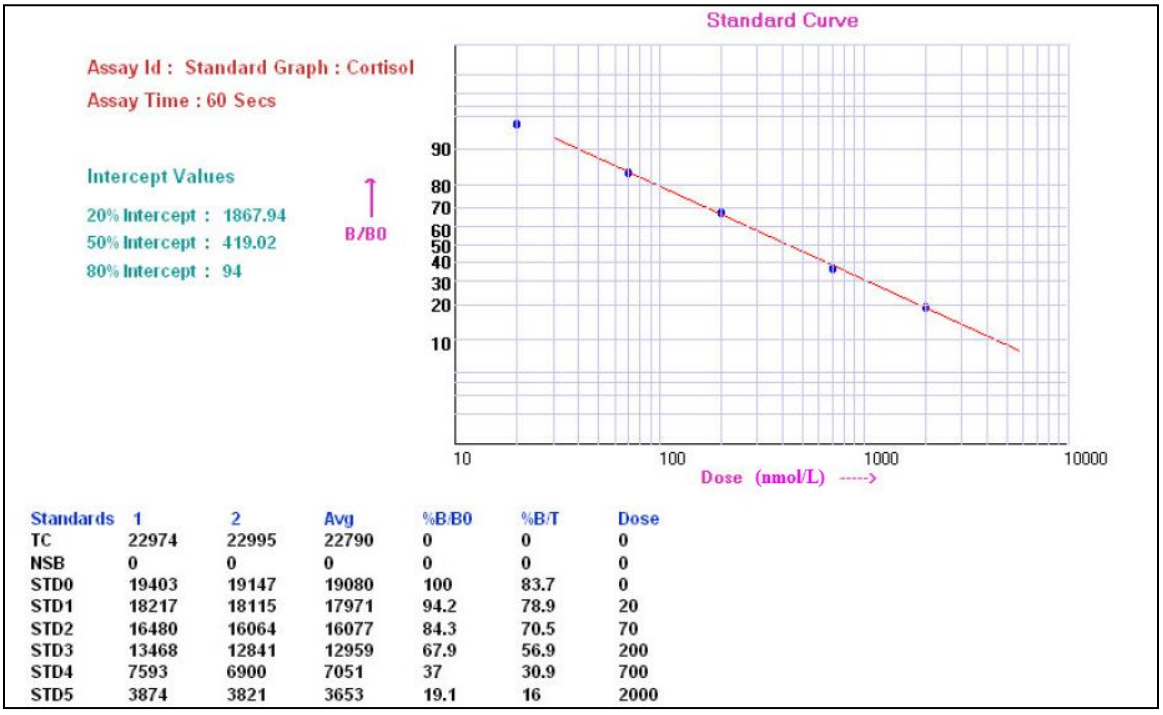
Standards	1	2	Avg	%B/B0	%B/T	Dose
TC	3252	2425	2652	0	0	0
NSB	760	898	642	0	24.2	0
STD0	16422	17457	16111	100	607.5	0
STD1	13901	7772	10007	62.1	377.3	2.5
STD2	6877	9062	7141	44.3	269.3	5
STD3	6955	7229	6263	38.9	236.2	10
STD4	3813	5854	4005	24.9	151	20

Appendix 7. Standard curve for radioimmunoassay of plasma insulin



Standards	1	2	Avg	%B/B0	%B/T	Dose
TC	26190	28207	27080	0	0	0
NSB	1919	1919	1801	0	6.7	0
STD0	9529	9529	7610	100	28.1	0
STD1	9027	9027	7108	93.4	26.2	7.5
STD2	8550	8550	6631	87.1	24.5	12.5
STD3	7595	7525	5641	74.1	20.8	25
STD4	6090	5942	4097	53.8	15.1	50
STD5	5447	5200	3405	44.7	12.6	100
STD6	3971	3971	2052	27	7.6	200

Appendix 8. Standard curve for radioimmunoassay of plasma cortisol



Standards	1	2	Avg	%B/B0	%B/T	Dose
TC	22974	22995	22790	0	0	0
NSB	0	0	0	0	0	0
STD0	19403	19147	19080	100	83.7	0
STD1	18217	18115	17971	94.2	78.9	20
STD2	16480	16064	16077	84.3	70.5	70
STD3	13468	12841	12959	67.9	56.9	200
STD4	7593	6900	7051	37	30.9	700
STD5	3874	3821	3653	19.1	16	2000